

Developing optogenetics for use in vascular research

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Abstract

Optogenetics is a recently established experimental technique that involves the heterologous expression of light sensitive proteins (opsins) in mammalian cells to modulate cell function. One of the most commonly used opsin is the blue light gated depolarising channel, channelrhodopsin-2 (ChR2). Optogenetics involving ChR2 has revolutionised the field of neuroscience by enabling the definition of novel brain circuitries. Optogenetic control of the electromechanical coupling in vascular smooth muscle cells (SMCs) is now emerging as a powerful research tool with potential applications in drug discovery and therapeutics. However, the exact ionic mechanisms involved in this control remain unclear. The overall aim of this thesis was to establish ChR2 for use in vascular optogenetics. The main findings of this thesis are:

- 1) Blue light activation of ChR2 and the ChR2 variant ChR2(H134R) led to long-lasting and non-inactivating depolarising currents.
- 2) Transgenic mice expressing ChR2(H134R) selectively in SMCs were generated. Isolated SMCs obtained from these mice demonstrated blue light induced depolarising whole-cell currents. Fine control of artery tone was attained by

varying the intensity of the blue light stimulus. This arterial response was sufficient to overcome the melanopsin-mediated light-dependent arterial relaxation observed in the presence of contraction-eliciting agonists.

- 3) Pharmacological analyses revealed that Ca^{2+} entry through voltage-gated Ca^{2+} channels, and recruitment of plasmalemmal depolarising channels (TMEM16A and TRPM) and intracellular IP_3 receptors were mandatory for the ChR2(H134R)-mediated arterial response to blue light at intensities $< \sim 0.1 \text{ mW/mm}^2$. Light stimuli of greater power evoked a significant Ca^{2+} influx directly through ChR2(H134R) and produced dramatic intracellular alkalinisation of the SMCs. The light intensity range that enables optical control of arterial tone primarily through the recruitment of endogenous channels and without substantial alteration of intracellular pH, was identified. Within this range, mice expressing ChR2(H134R) in SMCs are a powerful experimental model for achieving accurate and tuneable optical voltage-clamp of SMCs and finely-graded control of arterial tone, offering new avenues to the discovery of vasorelaxing drugs.
- 4) The Cl^- -selective ChR2 mutant iChloC mediates depolarising currents while also preventing H^+ or Ca^{2+} fluxes. This mutant was also found to have enhanced sensitivity to blue light. This new optogenetic tool could be a good candidate for an improved mouse model of vascular optogenetics.

To conclude, the work presented in this thesis represents an in depth analysis of the use of ChR2(H134R) in vascular optogenetics. The cellular mechanism linking ChR2(H134R) opening with contraction of vascular SMCs was defined. Additionally, the limitations of the current optogenetic mouse model have been identified, and

iChloC was shown to resolve these limitations. This study established vascular optogenetics as novel research tool vascular physiology and pharmacology.

Declaration

No portion of the work presented in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

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Thirdly, I would like to thank my supervisor Paolo Tammaro for his help and input over the course of this adventure. I am happy to say that, in addition to teaching and guiding me, Paolo has helped me understand some key Italian phrases and words that are highly useful in the high-pressure world of science.

Throughout this DPhil I have had the pleasure to interact with a huge number of people who have enriched my DPhil experience. If I were to thank them all individually there would be little time to write the rest of the thesis. Thank you all.

Abbreviations

AD/DA	Analogue-to-digital and digital-to-analogue
ADH	Anti-diuretic hormone
ADP	Adenosine diphosphate
AE	Anion Exchanger
Ani9	2-(4-chloro-2-methylphenoxy)-N-[(2-methoxyphenyl)methylideneamino]-acetamide
ANP	Atrial natriuretic peptide
ATII	Angiotensin II
ATP	Adenosine triphosphate
BK _{Ca}	Large conductance Ca ²⁺ -activated K ⁺ channels
[Ca ²⁺] _c	Cytosolic Ca ²⁺ concentration
CaM	Calmodulin
Ca ²⁺ -CaM	Ca ²⁺ -bound calmodulin
CaCC	Ca ²⁺ -activated Cl ⁻ channel
CaMK	Calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanosine monophosphate
ChR2	Channelrhodopsin-2
CICR	Ca ²⁺ -induced Ca ²⁺ release
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole

DIDS	4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid
DM	Dissociation medium
DMSO	Dimethyl-sulphoxide
EC	Endothelial cell
EDHF	Endothelial derived hyperpolarising factors
EDRF	Endothelial derived relaxing factors
EEA	Epoxy-eicosatrienoic acid
EGFR	Epidermal growth factor receptor
EL ₅₀	Light intensity causing half-maximal response
E _{rev}	Reversal potential
ET-1	Endothelin-1
G	Conductance
G _{max}	Whole-cell Ca ²⁺ conductance
h	Slope factor
HR	Halorhodopsin
HEK-293T	Human embryonic kidney 293T
I	Current
I _p	Peak current density
I _{CaCC}	CaCC current
I _{max}	Maximal asymptotic current
IP ₃	Inositol triphosphate
IP ₃ R	IP ₃ -sensitive receptor
[K ⁺] _o	Extracellular K ⁺ concentration
K _{ATP}	ATP-sensitive K ⁺ channel
K _{Ca}	Ca ²⁺ -activated K ⁺ channel

K _{ir}	Inward rectifying K ⁺ channel
K _v	Voltage-gated K ⁺ channels
LB	Luria-Bertani
LVGCC	L-type voltage gated Ca ²⁺ channel
MA	Mesenteric artery (<i>A. mesenterica</i>)
MAPK	Mitogen-activated protein kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MONNA	N-((4-methoxy)-2-naphthyl)-5-nitroanthranilic acid
NAADP	Nicotinic acid adenine dinucleotide phosphate
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide-binding domain
NCE	Na ⁺ /Ca ²⁺ exchanger
NA	Norepinephrine or noradrenaline
NKCC	Na-K-Cl cotransporter
NKE	Na ⁺ /K ⁺ exchanger
NMDG	N-methyl-D-glucamine
NO	Nitric oxide
OD	Optical density
PA	Pulmonary artery (<i>A. pulmonalis</i>)
PBS	Phosphate buffered saline solution
PCR	Polymerase chain reaction
PE	Phenylephrine
PGI ₂	Prostacyclin

pH _i	Intracellular pH
pH _e	Extracellular pH
P _i	Inorganic phosphate
PI	Phosphatidylinositol
PIP	Phosphatidylinositol-4 phosphate
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PIPK	PIP 5-kinase
pK _a	Apparent dissociation constant
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLB	Phospholamban
PLC	Phospholipase C
PMCA	Plasmalemmal Ca ²⁺ -ATPase
PSS	Physiological salt solution
R	Intracellular fluorescence ratio
R _{max}	Maximum fluorescence ratio
R _{min}	Minimum fluorescence ratio
ROC	Receptor operated channel
ROS	Reactive oxygen species
RSB	Retinal Schiff base
S _a	Slope factor of activation
SERCA	Sarcoplasmic/endoplasmic reticulum Ca ²⁺ -ATPase
S _h	Slope factor of steady state inactivation
SMCs	Smooth muscle cells

SOCE	Store-operated Ca^{2+} entry
STIC	Spontaneous transient inward current
STIM	Stromal interaction molecule
STOC	Spontaneous transient outward current
SR	Sarcoplasmic reticulum
TMD	Transmembrane domain
T_n	Normalised tension
TPC	Two-pore channel
TRP	Transient receptor potential
T_{ss}	Steady state tension
T_{ssn}	Normalised steady state tension
TxA_2	Thromboxane
TVGCC	T-type voltage-gated calcium channel
$V_{0.5,a}$	Midpoint voltage for current activation
$V_{0.5,h}$	Midpoint voltage for current inactivation
V_{hold}	Holding potential
V_m	Membrane potential
V_{max}	Maximal asymptotic V_m reached
V_{rest}	Resting V_m in the absence of blue light

Chapter 1

Introduction

This chapter aims to provide a broad overview of the general physiology of the vascular system. This is followed by a review of the endogenous ion channels that participate to the control of vascular tone. An overview of the use of exogenous light-activated ion channels to control cell function (an approach termed *optogenetics*) is also included as the primary aim of my DPhil project was to establish optogenetics as a novel tool in fundamental and applied vascular research. The specific objectives of this thesis are given at the end of this chapter.

1.1 *The vascular system*

The vascular system of vertebrates encompasses the pulmonary and the systemic circulation. In simple terms, the pulmonary circulation transports deoxygenated blood from the heart to the lungs and oxygenated blood back to the heart where it enters the systemic circulation [1,2]. The systemic circulation provides the rest of the body with oxygenated blood and brings deoxygenated blood back to the heart [3]. Arteries, the vessels carrying blood away from the heart, can be divided into subtypes. These include: conduit arteries, large arteries (which branch out from conduit arteries), feeding arteries and arterioles. The arterioles in turn subdivide into capillaries, which constitute the site for exchange of gasses and nutrients [3,4]. Capillary beds converge to form venules that in turn combine to form veins eventually merging into the *vena cava* [1,3,5]. The histology of the different blood vessels varies depending on the functional requirements of the vessel type. For example, arteries that carry blood at high pressure have thicker, more elastic walls compared to veins that carry blood at significantly lower pressure.

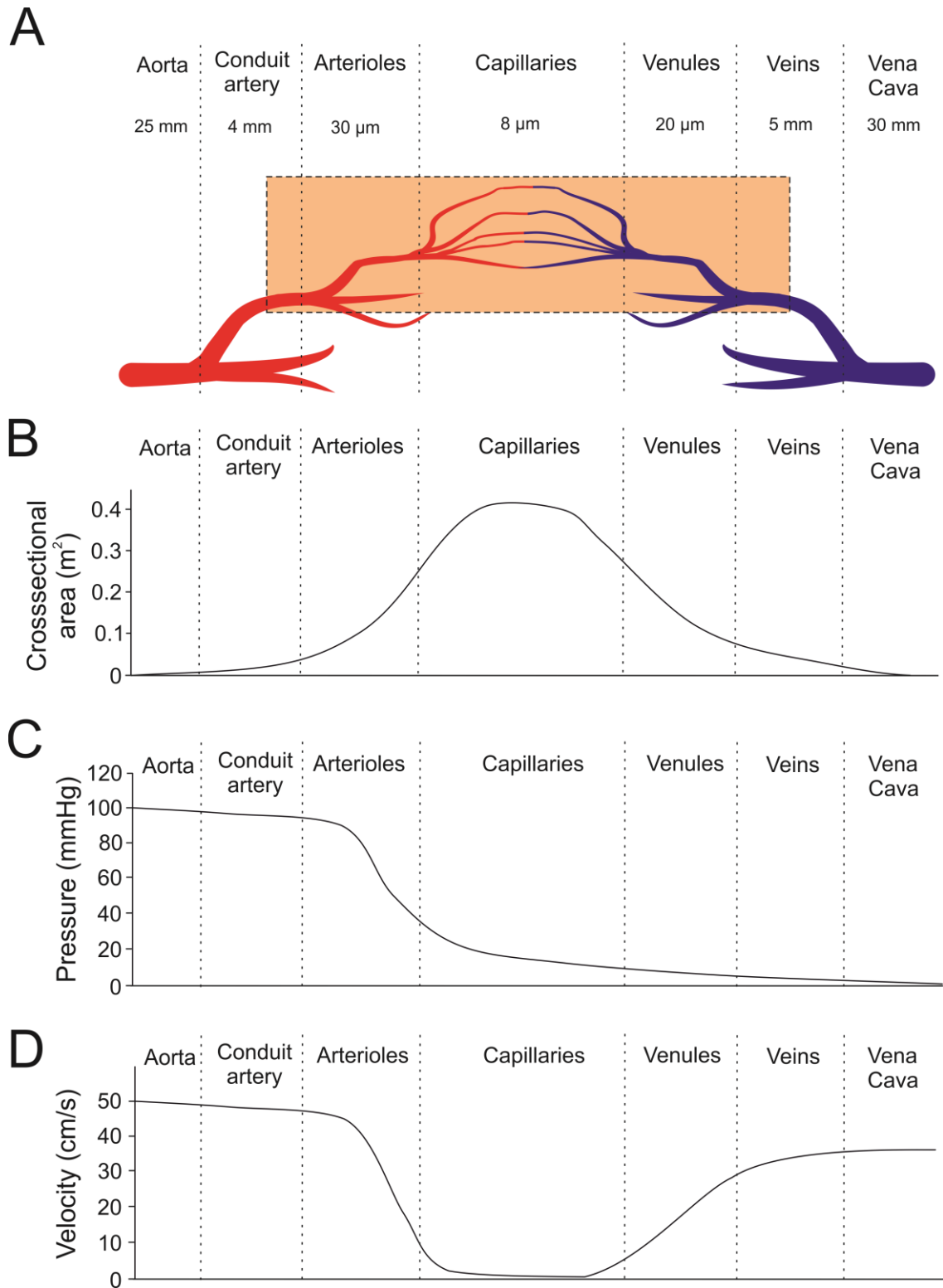


Figure 1.1. Relationship between the vessel type, total cross-sectional area, blood pressure and blood velocity.

A. Overview of blood vessel branching in the vascular system. For clarity, the branching is only shown for one vessel per branch point. The average blood vessel diameter (in humans)

*of each vessel type is displayed above the diagram. The orange box indicates the blood vessels involved in organ blood perfusion. **B.** Overview of how the total cross-sectional area of all the blood vessels of a certain classification varies across the circulatory system. **C.** Simplified schematic of the blood pressure in the different vessel types. **D.** Changes in blood velocity across the different blood vessel types. Both the velocity and pressure of the blood varies in the arteries during systole and diastole as the heart beats; for simplicity only the mean values are displayed. Composite figure adapted from [6].*

1.1.1 Blood vessel types

Arteries

In general, the diameter and wall thickness of blood vessels decreases as the distance from the heart increases (and blood pressure decreases) (Fig 1.1). The aorta is the largest artery, with a diameter of ~25 mm in humans, and is able to withstand high pressures (~120 mmHg in humans) as it carries the blood away from the left ventricle and into the systemic circulation. Arteries such as the aorta and the subclavian artery represent the major distensible arteries. They are high in elastin when compared to other vessels and therefore play a major role in elastic recoil during diastole [7]. This stretch allows energy to be stored in the artery wall which is then released during diastole. This elastic recoil alleviates large fluctuations in blood pressure between systole and diastole thus producing a smoother continuous flow of a more steady pressure [1–3]. This is termed the *Windkessel* effect. The conduit arteries also contain non-elastic fibres (collagen) to prevent overextension in response to increases in blood pressure [1,2,8].

Downstream of the main conduit arteries are distributing arteries that direct blood to the major organs in the body [7]. The distributing arteries have a thick muscular wall (with a thick, smooth muscle cell (SMC)-rich tunica media), but a reduced elastin content and do not always present an external elastic membrane [4]. Distributing

arteries, therefore, have a reduced capability to stretch in response to fluctuating changes in blood pressure, compared to the main conduit arteries [3,9,10]. The combination of low elastin and high collagen results in distributing arteries being less distensible (i.e. they are less compliant) [1,2]. Compliance can be defined as

$$\text{Compliance} = \frac{\Delta V}{\Delta P}, \quad \text{Eq. [1.1]}$$

where ΔV and ΔP refer to a change in arterial blood volume and a change in arterial blood pressure, respectively [8]. While the distributing arteries are contractile, they do not significantly contribute to redirecting blood flow to different organs; this is done primarily by the resistance arteries that branch off the distributing arteries [2]. The resistance arteries decrease in diameter and branch into resistance arterioles [2,3,10]. Arterioles present a wall thickness of $\sim 6 \mu\text{m}$ and measure only $\sim 30 \mu\text{m}$ in diameter in humans. Despite the thinner overall wall thickness and narrower vessel lumen, smaller arteries have a larger proportion of smooth muscle and less elasticity than the aorta. As explained in greater detail below, these small arteries provide large resistance to blood flow and therefore blood pressure decreases as the blood flow through these vessel types [2,3,5,6].

Capillaries

Capillaries are the smallest blood vessels ($\sim 8 \mu\text{m}$ in diameter in humans) [7]. The blood pressure in capillaries is low compared to the arterioles. This prevents rupturing of the thin capillary wall. Note, however, that in some bodily structures, such as the kidney's Bowman capsule, capillary pressure is elevated ($\sim 40\text{-}50 \text{ mmHg}$) to favour the process of filtration [3,5,6]. Another general feature of the capillary beds is that their overall cross sectional area is very high (Fig. 1.1).

Because the blood velocity is inversely propositional to the cross-sectional area, the speed of blood in capillaries is reduced. The reduced blood velocity favours gas and nutrient exchange between the blood and surrounding tissues. In general, a thin wall also aids in the diffusion rate of gases, nutrients and waste products between the capillaries and the neighbouring cells [5,6]. Some organs, such as the pancreatic islets and the kidney (in the glomerulus), have capillaries that have a fenestrated endothelial layer (from the latin *fenestra*, meaning window; openings in the endothelial layer which aids in the diffusion of small molecules and proteins). An image of a capillary section is shown in Figure 1.2.

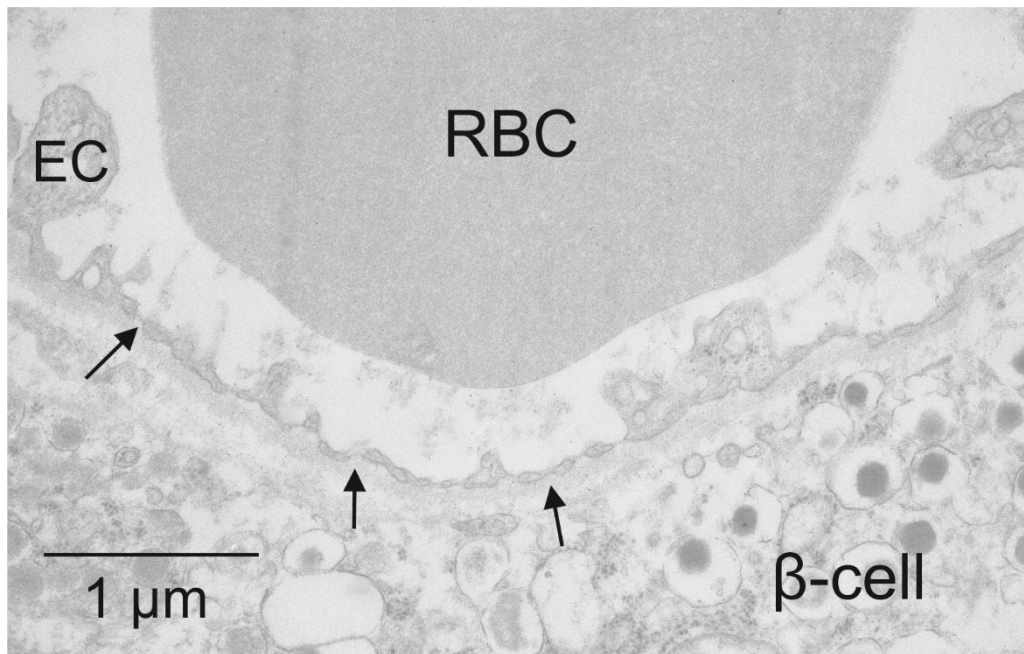


Figure 1.2. Electron microscopy of a capillary passing through a pancreatic islet of Langerhans.

The red blood cell (RBC) travels through the lumen of the capillary, which is made up of a very thin endothelial cell (EC) monolayer. The thinness of this layer reduces the diffusion distance from the capillary to the tissue cells. The fenestration in the EC layer (as indicated by the arrows) also allows the passage of small proteins. Acquired by NJG Rorsman under the supervision of Dr A Clark, University of Oxford.

The wall thickness of a blood vessel directly relates to the amount of pressure it can withstand. This is expressed by the Laplace law, which relates tension, pressure and radius of the vessel as:

$$Tension = P_i \times r, \quad \text{Eq. [1.2]}$$

where tension is the wall tension (the force per unit length of the vessel wall), P_i is the effective transmural pressure (in mmHg) of the blood vessel (essentially equal to the intraluminal pressure as the extravascular pressure is minimal) and r is the radius (in mm) of the blood vessel. Thus, for any given pressure, increasing the radius of the blood vessel results in a linear increase in tension. This explains why arteries require thick walls to carry blood at a high pressure through a wide lumen. It follows from this that tension in the capillary wall is low (because of their small radii) and they are therefore capable of withstanding relatively high blood pressures (~20-40 mmHg) in spite of its extremely thin wall consisting of an endothelial cell monolayer [2,6].

Venous blood vessels

Blood flows through the venous circulation in increasingly large vessels finally reaching the heart. Due to the low pressure in the venous circulation, vessels with similar lumen diameter as arteries have comparatively thin walls. The smallest venous vessel are the post-capillary venules, which arise from the merger of capillaries leaving a capillary bed. These post-capillary venules have an endothelial layer and a thin layer of connective tissue. They combine to form larger diameter venules that exhibit a thin muscular layer that can regulate the capillary blood flow by contracting or dilating. Venules drain into veins. Veins carry blood back towards

the heart at low pressure. Veins have valves that prevent the flow of blood to go in the reverse direction [2,8]. The combination of these valves with the contraction of nearby skeletal muscle (e.g. in the lower limbs) aids the return of blood by the “skeletal muscle pump” process [6]. Compared to arteries, veins have significantly thinner and less muscular walls [3]. As a result of the thin elastic walls, veins are extremely compliant and can undergo significant stretch [2]. This stretch, combined with the larger lumen size of veins compared to arteries means that at any time >60% of the total blood volume is in the venous part of the circulation, allowing the systemic veins to act as a blood reservoir [2]. Despite the low pressure, vein still have muscular walls. The role of the muscle in the wall is to contract to reduce the lumen diameter and aid the return of blood to the heart, thus increasing the cardiac output to maintain the arterial pressure in the event of blood loss [2,5].

1.2 *Haemodynamics*

Blood flow and pressure changes across the circulatory system (Fig. 1.1). Factors influencing these changes include volume and viscosity of the blood, cardiac output, blood vessel compliance, and blood vessel length and diameter [6]. Each of these factors affect the blood flow through regulating the resistance of the circulatory system [1,2].

The resistance of the blood flow through a single blood vessel is described by the Poiseuille equation:

$$F = \frac{\Delta P \times \pi \times r^4}{8 \times \eta \times l}, \quad \text{Eq. [1.3]}$$

where F is the blood flow, ΔP is the change in perfusion pressure from the entrance and exit of the blood vessel, r is the vessel radius, η is the viscosity of the blood and l is the vessel length [2].

The Poiseuille equation is based on some assumption such as the fact that the fluid is Newtonian (i.e. the viscosity is constant and the flow is uniformly laminar as opposed to being turbulent in certain regions of the vasculature), and the vessel is straight and rigid. In spite of the fact that these conditions do not always apply *in vivo*, this equation is of great importance in vascular physiology as it shows that the main factor regulating the resistance of the vessel is its radius. A change in blood vessel radius causes a change in the blood flow to the forth power. Thus, halving the artery radius results in a 16-fold reduction in flow.

In vivo blood vessels are either in series or parallel configuration. This affects the overall resistance to blood flow produced by an entire vascular bed. The overall resistance of vessels arranged in series is the sum of the individual resistances

$$R_{ser} = R_1 + R_2 + R_3 + \dots R_n \quad \text{Eq. [1.4]}$$

where R_{ser} is the total series resistance and R_1, R_2 etc. are the resistance of the individual resistors (blood vessels) in the resistance circuit. R_n is the resistance of the n^{th} vessel.

In contrast, the total parallel resistance (R_{par}) is given by

$$\frac{1}{R_{par}} = \frac{1}{R_1} + \frac{1}{R_2} + \frac{1}{R_3} + \dots \frac{1}{R_n} \quad \text{Eq. [1.5]}$$

Thus, the parallel configuration of blood vessels has a lower overall resistance (the sum of the resistances) than a serial configuration. Changing the resistance of a single vessel (resistor), will have little effect on the total resistance of the system. Vessels of one type are generally arranged in parallel while vessels of different types are organised in series. For example, the distributing arteries draw blood from an elastic artery in series with "resistance vessels" (including small arteries and arterioles), which are then, in turn, disposed in parallel [2,3].

1.3 Blood vessel histology

Blood vessels typically comprise three primary layers (tunicae): the tunica intima, tunica media and tunica adventitia (Fig. 1.3).

Tunica intima

The tunica intima is the innermost layer and lines the lumen. It consists of a monolayer of ECs, separated from the tunica media by a subendothelial layer. In larger arteries there is also an internal elastic lamina (which helps to facilitate the elastic recoil as the pressure decreases following diastole) [1,2,8]. The EC layer is separated from the surrounding tissue by a basement membrane consisting of collagen, fibronectin, laminin and amorphous ground substance [1,3,11]. The tunica intima forms the primary blood barrier interface and facilitates smooth blood flow, inflammatory responses and regulates haemostasis. Additionally, the endothelial layer influences artery tone through the release of factors such as nitric oxide (NO), a vasodilator, that act on SMCs in the tunica media [12,13]. Both capillary and venule walls contain a population of pericytes. These cells are involved in regulating

angiogenesis and in the neurovascular coupling, and are positioned intermittently along the length of the vessel [14,15]. Pericytes are also contractile and are involved in the fine control of blood perfusion in many organs as described in greater detail below (see Section 1.4.2).

Tunica media

The tunica media is formed from layers of SMCs and elastic laminae [16]. These laminae are connected by elastic fibres [16]. The number of parallel sheets of elastic laminae and SMCs is typically higher in vessels experiencing a higher transmural pressure [16]. The contractile mechanisms in SMCs and its control are described in further detail below. The external elastic lamina, like the internal elastic lamina smooths the blood flow by allowing elastic recoil [1,2,8,16]. Venules and capillaries lack a tunica media [1,2,8,16].

Tunica adventitia

The outermost layer of blood vessels is the tunica adventitia. This is composed of an external elastic lamina (made up of elastin), collagen and connective tissue fibres that anchors the blood vessel to the surrounding tissue. Large conduit arteries exhibit a greater degree of compliance than smaller resistance arteries [4]. However, continuous strain results in elastin becoming damaged leading to a weakening of the arterial wall. To combat this, a large amount of collagen is present in the vessel wall to provide additional structural support. The collagen prevents vessel wall collapse in response to external pressure as well as overextension of the elastin in conduit arteries. This is particularly important within the aortic arch where the blood pressure in man is between 80 and 120 mmHg (diastole and systole, respectively) [1,2,8]. Within the adventitia there are also a number of cells

such as fibroblasts, whose role is to synthesise extracellular matrix, and perivascular nerves involved in sympathetic innervation. The innervation of the adventitia by sympathetic neurones allows the vascular tone to be controlled through release of neurotransmitters, which acts on the SMCs in the tunica media. In large vessel, there are also small vessels within the blood vessel wall (*vasa vasorum*) whose role is to supply the vascular tissue with O₂ and nutrients [17,18]. The adventitia also harbours T-cells, macrophages and progenitor cells [5,10,11,19].

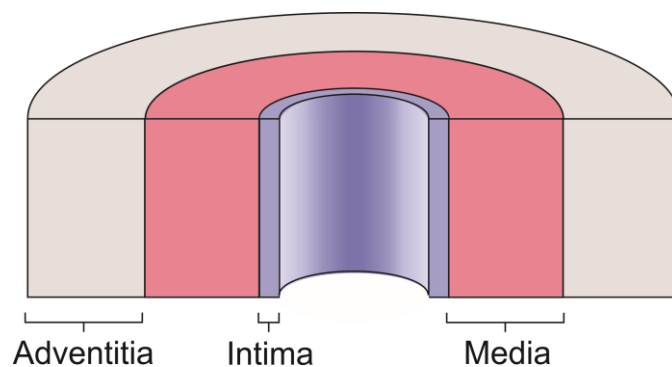


Figure 1.3. The structure of the artery.

Blood vessels consist of a tunica intima, a tunica media and a tunica adventitia (as indicated). Each tunica has multiple components that have been omitted for clarity.

1.4 **Blood vessel cytology**

1.4.1 *Endothelial cells*

Endothelial cells (ECs) are thin (<0.3 μm [20]) and elongated (>200 μm long [21], <10 μm wide [22]) cells that form a semi-selective barrier. They form a smooth surface that provides an anti-thrombolytic barrier that controls the passage of immune cells into and out of the vessel whilst inhibiting blood clotting [15]. ECs modulate vessel tone via synthesis and release of several vasoactive substances

(such as endothelin-1 and nitric oxide (NO)) in response to neurotransmitters, mechanical stress, cytokines, and platelet-derived factors [12,23]. In addition to this “chemical” coupling an electrical coupling between SMCs and ECs exists through the formation of gap junctions [24]. Gap junctions in the cardiovascular system are assembled primarily by connexin (Cx) proteins [24]. The connexin proteins present vary between different vessel types as well as between the SMCs and ECs [24,25]. The predominant connexin between SMCs is Cx43 but other Cx proteins are also present at varying degrees. In contrast, the main Cx forming gap junctions between ECs and SMCs is Cx40 [26]. This allows hyperpolarisation to spread from ECs to adjacent SMCs, inhibiting contraction [27].

The cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) plays a major role in governing EC functions, including the production and release of vasoactive substances. Increases in $[\text{Ca}^{2+}]_c$ is primarily achieved through Ca^{2+} release from intracellular stores (chiefly via the inositol trisphosphate receptor (IP_3R) but also, albeit to a lesser extent, the ryanodine receptor (RyR) [28]) in response to G-protein couple receptor (GPCR) signalling, or via stretch-activated channels within the plasma membrane [13,29,30]. The increase in $[\text{Ca}^{2+}]_c$ leads to cell hyperpolarisation due to the activation of Ca^{2+} activated K^+ (K_{Ca}) channels [31–33]. This promotes Ca^{2+} influx through receptor- and store-operated channels by increasing the electrical driving force. This increase in $[\text{Ca}^{2+}]_c$ leads to Ca^{2+} -calmodulin (Ca^{2+} -CaM) formation and thereby stimulate production of the vasodilator NO by activation of the endothelial nitric oxide synthase (eNOS) [13]. eNOS activity is also regulated by a variety of other proteins (e.g. heat shock protein 90 [34]) and post translation modifications (e.g. phosphorylation [35]), as reviewed in [36].

1.4.2 Pericytes

Pericytes are cells present on precapillary arterioles, capillaries and venules. Pericytes play multiple roles, including in angiogenesis and maintenance of the blood brain barrier [14]. Importantly pericytes are contractile and can control blood perfusion by directly modulating the capillary diameter. Their role in this respect is especially well defined in the brain, where pericytes are crucial for neurovascular coupling [37]. Alteration in pericyte function is also known to be implicated in the no-reflow phenomenon in stroke [38]. Following stroke, the pericytes die and remain in a state of rigor; this prevents blood supply to the downstream regions. Pericytes may be involved in a similar maladaptive response in the heart as the microcirculation is irrecoverably affected following myocardial infraction [39].

1.4.3 Vascular smooth muscle cells

Vascular SMCs in the tunica media are elongated cells that are $\sim 5\text{-}10\ \mu\text{m}$ in width and $50\text{-}150\ \mu\text{m}$ long [1,40,41]. As shown in Figure 1.4 these cells are organised either end to end, forming a continuous ring of SMCs in parallel to elastic laminae (*Ringmuskeln*), or connecting concentric elastic laminae (*Spannmuskeln*) [42].

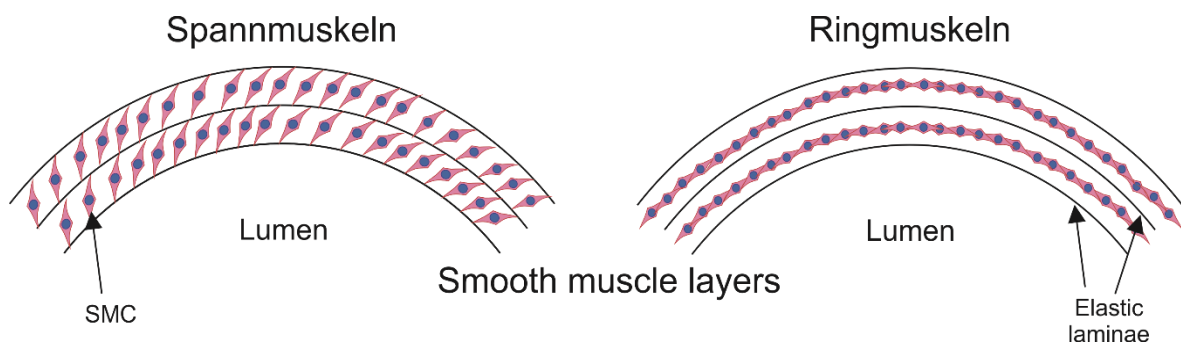


Figure 1.4. Smooth muscle cell arrangement in the arterial wall.

Sections of the artery wall, highlighting only the tunica media, are shown. Spannmuskeln is found in the large conduit arteries, whereas the Ringmuskeln configuration is found in distributing arteries and arterioles. Adapted from [42].

The main function of vascular SMCs is to regulate blood flow to tissues and organs via contraction and altered blood vessel diameter [1,2]. The absence of striation in smooth muscle is due to the disorganised nature of the sarcomeres (that are organised in parallel in striated muscle) [2,10]. SMCs in the vasculature exhibit numerous invaginations of the plasma membrane (caveolae). The principal role of these caveolae is to facilitate cell signalling. For example, many GPCRs and ion channels congregate to caveolae [2,3,43]. Caveolae are also involved in the crosstalk between the sarcoplasmic reticulum (SR) and the plasma membrane (PM) by positioning the two membranes in close proximity. This allows signalling events in either cellular component to be relayed quickly to the other compartment. For example, G_q -PCR signalling leading to Ca^{2+} release via activation of SR bound inositol-triphosphate receptor (IP_3R) can lead to activation of plasmalemmal ion channels (such as the Ca^{2+} activated Cl^- or K^+ channels) [10,44]. Additionally, the invaginations constituted by the caveolae, may result in localised domains where the composition of the extracellular medium differs from that in general [45,46]. Such changes can affect ion transport across the membrane due to localised changes in membrane potential (V_m) and/or ionic composition.

1.5 *Excitation-contraction coupling in the cardiovascular system*

As is the case in other muscle types, smooth muscle contraction requires interaction between actin and myosin [2,3,5,47]. Actin associated proteins such as caldesmin,

paxillin and calponin also play a role in SMC contraction [48]. SMC contraction is downstream of increases in $[Ca^{2+}]_c$ [1,2]. The amount of force generated, and thus the SMC contraction observed, is finely controlled by regulating the $[Ca^{2+}]_c$ and is coupled to changes of the V_m via the activity of SMC L-type voltage gated Ca^{2+} channels (LVGCCs) [3,8,49]. The coupling between the V_m and the contractile state is termed the excitation-contraction coupling.

1.5.1 Mechanisms of contraction and relaxation of SMCs

Increases in $[Ca^{2+}]_c$ leads to a conformational change in calmodulin (CaM) through the binding of four Ca^{2+} ions. The Ca^{2+} -bound CaM (Ca^{2+} -CaM) activates myosin light chain kinase (MLCK) which in turn phosphorylates myosin light chain (MLC) subunits (a process requiring ATP). MLC is non-covalently bound to myosin heavy chain (MHC) and regulates the interaction between the MHC head-group and the actin filaments via MHC ATPase activity [47,50]. In the resting state, the affinity of the myosin headgroup for the actin filament is low due to the binding of ATP. Once MLC is phosphorylated, ATPase activity of the myosin head group is increased, leading to ATP hydrolysis and formation of ADP and inorganic phosphate (Pi). ADP and Pi remain bound to the myosin head group and induce a conformational change that increases the affinity of the myosin for actin and thus promotes myosin-actin binding. The cross bridge formation between the myosin heads and the actin filaments causes contraction due to the ratcheting of the myosin and actin filaments [2,3,10].

In comparison to striated muscle, the contraction of vascular smooth muscle is slow. This is in part because the myosin variant in smooth muscle has a slower ATPase activity, ATP-induced dissociation, as well as a lower rate of crossbridge formation

than striated muscle [51]. Furthermore, in striated muscle, the resting state of the myosin filament is already bound to ADP and Pi and is ready to contract (they are in a “cocked” state) once the $[Ca^{2+}]_c$ rises to induce troponin-tropomyosin conformational change leading to the uncovering of the actin binding sites [52]. In smooth muscle, myosin remains associated with ATP until MLC is phosphorylated and it is not necessary for the actin binding site to be covered when in the unstimulated state [52]. The requirement of an activating phosphorylation of MLC increases the time necessary to initiate SMC contraction.

Once the contractile stimulus (e.g. from NA stimulation) ceases, Ca^{2+} is removed from the cytoplasm by the plasmalemmal and sarcoplasmic reticulum Ca^{2+} ATPases (PMCA and SERCA) and the Na^+/Ca^{2+} exchanger (NCE). Decrease in $[Ca^{2+}]_c$ leads to reduced Ca^{2+} -CaM and reduced MLCK activity. When $[Ca^{2+}]_c$ falls below $\sim 0.1 \mu M$ [1], the binding of Ca^{2+} to calmodulin is decreased to the extent that MLCK activity ceases [1]. However, the relaxation of SMCs is also slow in comparison with that of cardiomyocytes and skeletal muscle. To induce relaxation, MLC needs to be dephosphorylated by myosin light chain phosphatase (MLCP). Once this occurs, myosin ATPase activity stops [53]. MLCP activity, in contrast to MLCK, is Ca^{2+} independent [54]. Thus, MLCP can act at any stage during the cross-bridge cycling process. Therefore, MLC dephosphorylation can occur at the end of the power stroke, which prevents the exchange of bound ADP for a fresh ATP (due to decreased ATP affinity). This allows the interaction between the myosin head group and the actin to remain in contact and thus the degree of contraction continues. This phenomenon is called “latching” and is dependent on the balance between the MLCP and MLCK activity. Low level of MLCK and MLCP activity is required for the maintenance of the latch state. Because of the latch mechanism, a

degree of contraction can be maintained for prolonged periods at low ATP consumption rates [2,3,10,55,56].

Other proteins, such as caldesmon and calponin, also participate in regulating the smooth muscle contraction. Caldesmon inhibits the ATPase activity of the myosin until acted upon by Ca^{2+} -CaM [57]. Calponin is an actin-associated protein, which inhibits the ATPase activity and crossbridge formation thus inhibiting smooth muscle contraction [58].

1.6 *Extrinsic and intrinsic control of vessel tone*

The control of blood flow, through the constriction and dilation of arterioles, plays a decisive role in the adjustment of blood flow to meeting the metabolic needs of the body. It is a highly regulated process. The factors that control the vessel lumen radius are categorised as either intrinsic or extrinsic mechanisms (Fig. 1.5) [10]. The degree of contraction of an individual arteriole is determined by the balance of the contractile and dilatory signals acting on it at any one time [2].

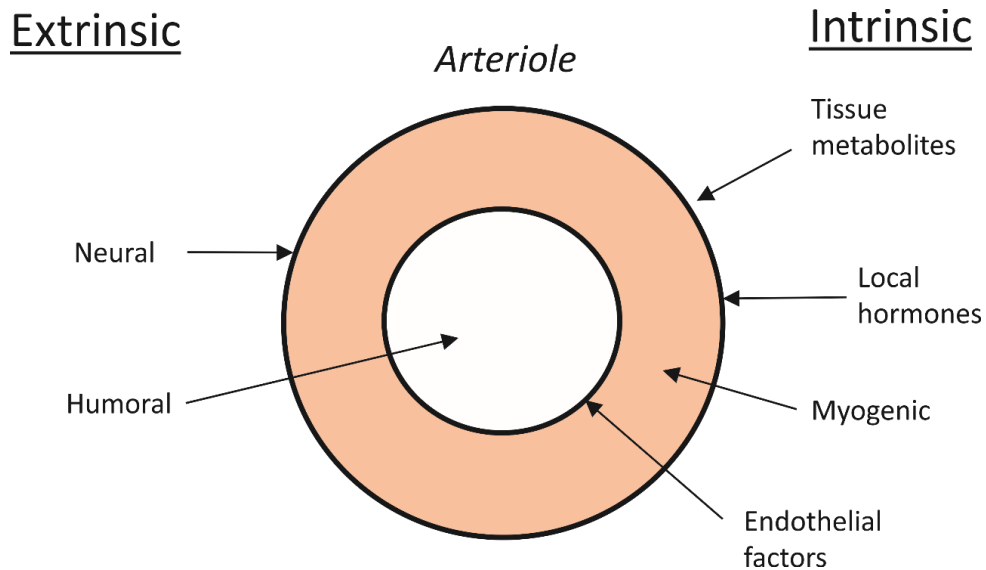


Figure 1.5. Overview of the intrinsic and extrinsic control mechanisms of arteriole tone. Adapted from [2].

1.6.1 Extrinsic

Extrinsic mechanism of blood vessel tone control includes neural innervation and circulating hormones and is usually considered to act on a more global scale, controlling the systemic vascular resistance [2].

The vasculature is primarily innervated by the sympathetic nervous system [2]. In broad terms, sympathetic signals are involved in the flight-or-flight response. Neurotransmitters (e.g. noradrenaline (NA)), released from the termini of the sympathetic neurones (found in the adventitia), diffuse through the extracellular matrix to bind to receptors in the SMCs plasma membrane. Different neurotransmitters activate distinct GPCRs and this allows a spectrum of responses. SMCs express α_1 , α_2 and β_2 -adrenoceptors. Activation of G_q coupled α_1 receptors leads to contraction following activation of the IP_3 signalling pathway [1,2,5]. Activation of the G_i coupled α_2 receptors also causes SMC contraction but via reduced formation of cAMP (which inhibits MLCK) [59]. Conversely, G_s coupled β_2

receptor activation stimulates relaxation by increasing adenylyl cyclase activity and thus the concentration of the MLCK cAMP [2,5,60]. Electrical coupling between SMCs via gap junctions allows the innervation of a single SMC to spread to neighbouring cells, resulting in a synchronised response to neural stimulation [5,10]. This allows the contractile state of multiple SMCs to be controlled by one neurone [3]. Resistance vessels are extensively innervated by autonomic nerves and thereby controls arterial tone [2,8].

Acetylcholine, released from ECs in response to shear stress (due to increased blood flow), bind to muscarinic acetylcholine receptors (G_q -PCRs) in an autocrine fashion leading to NO production following an IP_3R mediated increase in $[Ca^{2+}]_c$ [2,61,62].

Hormones (e.g. atrial natriuretic hormone (ANP), angiotensin II (ATII)) also bind to plasma membrane bound receptors, leading to activation of intracellular signalling pathways. ANP is released by atrial cardiomyocytes and travels through the circulation to the target cells (primarily in the kidney), where it causes dilation in afferent glomerular arterioles through an increase in cGMP within the SMCs [2,63]. This results in an increase in the capillary pressure, which in turn leads to an increase in the filtration rate (as more fluid is leaving the capillary through the fenestrated capillary wall). ATII is a hormone produced by vascular ECs from circulating angiotensin I [2]. It acts on G_q -PCRs on the SMCs, which leads to contraction due to IP_3 -induced increase in $[Ca^{2+}]_c$ [2,64].

1.6.2 *Intrinsic*

In contrast to the extrinsic control of artery tone, the intrinsic mechanisms are the principal determinants of local blood flow through vascular beds. The intrinsic

mechanisms of control include paracrine signalling through the release of endothelial-derived factors (e.g. endothelin-1 and NO [10,12,65]), myogenic effects, locally released hormones (e.g. histamine, bradykinin) and metabolic waste products (e.g. CO₂) [66,67]. Some of these are considered below.

Myogenic effect

A key intrinsic control mechanism is the myogenic (also known as Bayliss) effect [2,3]. This is characterised by a contraction of the SMCs following an increase in blood pressure. The change in vessel tone resulting from increased blood pressure is myogenic; i.e. it originates from within the vessel itself [2,3,68]. The mechanism behind it is not fully understood but is thought to be at least in part mediated via the activity of stretch- or volume-regulated channels (members of the TRP channel family, see Section 1.8.2) in the SMCs [69,70]. Activation of these channels results in membrane depolarisation, activation of LVGCCs, increased Ca²⁺ influx and elevation of [Ca²⁺]_c, culminating in SMC contraction [3]. There is also evidence for a role for volume-regulated Cl⁻ channels and Na⁺ channels in the myogenic response [71,72].

Vasomotion is the term given to the spontaneous rhythmic contractions of arterial walls. This cyclic contraction and relaxation involves the activation of CaCCs due to [Ca²⁺]_c sparks or waves initiated by IP₃R activity [73]. The depolarisation spreads between the SMCs, allowing the contraction to propagate. The role of vasomotion is not fully understood but it has been suggested that it promotes greater flow through the vessel [73,74]. Another theory is that the contraction and relaxation prevents the latching of the myosin fibres (due to the low affinity of ATP), thus allowing more a rapid response to subsequent contractile or dilatory stimulus. In

contrast to the peristaltic synchronicity observed in the rhythmic contractions of smooth muscle outside the circulatory system, vasomotion in blood vessels is coordinated over extended lengths of the vessel. ECs also play a role in regulating the myogenic tone, as described below. Thus, the myogenic response to alterations in blood flow is mediated by vasoconstrictive and vasodilatory mechanisms acting in concert originating from both ECs and SMCs.

Endothelium-derived control of vessel tone

Increases in blood pressure leads to both cross-sectional and shearing stress on the vessel wall. Flow-induced shear stress leads to ECs aligning with the direction of the flow, EC depolarisation, as well as changes in the expression of endothelin-1 (ET-1), eNOS and thrombomodulin (an anti-coagulant) [75–77]. eNOS produces NO from L-arginine and O₂ in a calmodulin-dependent process [78]. NO is a potent vasodilator, which diffuses from the ECs to the neighbouring SMCs where it promotes relaxation. The resultant vasodilation decreases the local blood pressure. Upregulation of eNOS expression in conjunction with upregulated eNOS activity result in the increased NO production and release from the ECs [76,79,80]. NO produced in response to the shear stress combats overconstriction from the myogenic response. The mechanism behind the NO-induced relaxation is driven by activation of cGMP-dependent protein kinase (PKG) in SMCs following an accumulation of cGMP due to an increase in the activity of the guanylyl cyclase enzyme (activated by NO) [2]. Activation of PKG (via increased cGMP levels) lowers contractility by a combination of effects: i) reduced IP₃R-mediated Ca²⁺ release from the SR [81]; ii) increased activity of myosin light chain phosphatase (MLCP); iii) reduced Ca²⁺ channel activity; iv) increased K⁺ channel activity [3,53,82]; and v)

increased activity of the plasmalemmal Ca^{2+} -ATPases (PMCA). In addition, NO suppresses the contractile effect of the vasoconstrictor endothelin-1 (ET-1) [82].

Bradykinin

Bradykinin is an inflammatory protein, which causes blood vessel dilation. Bradykinin acts via the G_q - or G_i -coupled bradykinin β_2 receptors on ECs [3]. This leads to NO production following an increase in the $[\text{Ca}^{2+}]_c$ [61,83]. Furthermore, bradykinin action leads to IK_{Ca} channel activation, leading to hyperpolarisation of the EC leading to further NO production due to increased Ca^{2+} influx [84]. Bradykinin plays an additional role in SMC proliferation by activating MAPK-dependent proliferative pathways [85].

Prostanoids

Prostanoids is a category of signalling molecules (that includes prostaglandins, thromboxanes and prostacyclins) that are all derived from arachidonic acid by cyclooxygenases [86],

Prostacyclin (PGI_2) is a vasodilator that is constitutively produced and released by vascular ECs, but further release is stimulated by inflammatory agents, leading to inflammatory vasodilation [3]. PGI_2 acts on platelets, SMCs and ECs through G_s -PCR signalling [2]. PGI_2 increases intracellular cAMP, which in turn activates PKA. PKA phosphorylates MLCK, thus reducing its activity, leading to a reduced degree

of SMC contraction. In addition to its vasodilatory effect, PGI₂ prevents platelet aggregation and thus reduces the incidence of thromboses.

Prostaglandins (e.g. PGE₂) are released by the SMCs and acts as a vasodilator in a paracrine and autocrine manner by binding to G_s-coupled receptors. In contrast, PGF₂ is a vasoconstrictor, binding to G_q coupled receptors on SMCs [2].

Thromboxane A₂ (TxA₂), released from platelets, SMC and ECs, acts on SMCs to promote vasoconstriction and blood clotting through platelet aggregation [87–92]. TxA₂ may act in a co-operative manner to enhance the response to serotonin (released from neurones innervating the blood vessels) to control artery tone, in particular in response to stenosis or tissue damage [2,93].

Endothelium-derived hyperpolarising factor

The endothelium-derived hyperpolarising factors (EDHFs) is a category of vasodilatory influences (excluding NO and prostacyclins) that emanate from the ECs that act on SMCs to cause SMCs hyperpolarisation, and thus relaxation and vasodilation [94]. The exact nature of EDHF was long debated, but it has become apparent that there are numerous mechanisms behind it that appear to vary in prominence between artery types and between species (reviewed in [95]). For example, endothelial intermediate and small conductance calcium activated potassium channels (IK_{Ca} and SK_{Ca}, respectively) contribute to EDHF currents, as was determined through the application of the SK_{Ca} and IK_{Ca} blockers apamin, TRAM-34 and charybdotoxin [96,97]. The efflux of K⁺ through these channels leads to EC hyperpolarisation which has the potential to be transmitted to the SMCs via the direct electrical coupling provided by the gap junctions present between ECs and SMCs. Furthermore, the efflux of K⁺ into the interstitial space can lead to an

increase in current mediated by SMC inward rectifying potassium (K_{ir}) channels leading to SMCs hyperpolarisation [98,99]. Additional SMC hyperpolarisation has also been reported as a consequence of the activation of the Na^+/K^+ exchanger (NKE) by increased extracellular K^+ concentration [100,101]. Metabolites of arachidonic acid, such as epoxy-eicosatrienoic acid (EEA), have also been proposed to act as an EDHF [3,27,102,103]. EEA-induced relaxation has been shown to be due to paracrine signalling (activation of SMC BK_{Ca} channels following EEA release from ECs) and/or EEA induced activation of EC transient receptor potential (TRP) channels and hyperpolarisation which spreads from the ECs to SMCs, with the mechanism varying between different artery types (reviewed in [94]).

Endothelin

ECs also produce and release contractile agonists such as the endothelin peptides. The most potent and abundant of the three endothelin peptides is endothelin-1 (ET-1) [104]. Endothelin receptors (the G_q -PCRs ET_A and ET_B) on the SMCs bind the locally released ET-1 which activates the IP_3 signalling pathway, leading to contraction due to release of Ca^{2+} from intracellular stores, $CaCC$ activation, depolarisation and LVGCC activation as previously described (Section 1.6.2) [3,105]. Additionally, this activates non-selective TRPC channels, resulting in Ca^{2+} influx and membrane depolarisation [3]. ET-1 also binds to ET_B receptors on ECs in an autocrine manner, which results in a vasodilatory response [2,106,107]. However, as soon as ET-1 acts on SMCs the dilatory stimulus is superseded by the contractile effect of ET-1 on SMCs [108]. The role of the autocrine signalling has also been implicated in EC growth and proliferation as well as EC-leukocyte

interaction [106,109]. In comparison to other contractile agonists (such as NA), ET-1 binding leads to prolonged activation of the depolarising, Ca^{2+} increasing effect, thus causing an extended period of contraction [3,106]. The potency and duration of ET-1 signalling means that dysregulation of ET-1 release has a profound impact on the resting arterial tone under pathological conditions [104]. The release of ET-1 is inhibited by NO, prostacyclin and ANP, while activated by ATII and antidiuretic hormone (ADH) [2].

1.7 *Electrical properties of membranes*

An electrical potential exists across all living cells due to the unequal distribution of ions between the cytosol and the extracellular environment. The resulting combined electrical and concentration gradients provide the electrochemical force that determine direction of ion fluxes through ion channels. Each ion tends to diffuse across the membrane until an electrochemical equilibrium (E) is established. This equilibrium is reached when the electrical and chemical forces balance. The value of E is given by the Nernst equation

$$E_{ion} = \frac{RT}{zF} \ln \frac{[X]_o}{[X]_i}, \quad \text{Eq. [1.6]}$$

where E_{ion} is the electrical potential for ion X, $[X]_i$ is the intracellular concentration of the ion, $[X]_o$ is the extracellular concentration of the ion, R is the universal gas constant, T is the temperature in Kelvin, z is the charge of the ion and F is Faraday's constant [110]. The physiological intracellular and extracellular ion concentrations and the calculated E_{rev} for the main physiologically relevant ions are displayed in Table 1.1.

Table 1.1. Intracellular and extracellular ion concentrations in vascular SMCs.

Ion	Intracellular (mM)	Extracellular (mM)	Relative ion permeability (pX/pK ⁺)	E _{rev} (mV)
K ⁺	140-150 [3,111,112]	5.5 [3,113]	1	-82
Na ⁺	10-20 [3,112,114]	135-140 [3,113]	~0.15-0.20 [60,115,116]	67
Cl ⁻	~40 [115,117–122]	115-129 [3,113]	~0.60 [116]	-30
Ca ²⁺	0.001-0.01 [10,112,123]	~1.28-4 [10,113,123]		146

Ion concentrations are given in mM (intracellular concentrations are primarily from aortic SMCs). The permeability of the ions is expressed relatively to the K⁺ permeability of the plasma membrane. The E_{rev} was calculated using the Nernst equation (Eq. 1.6).

A variety of ion channels specific for different types of ion, are present in the cell membrane. Thus, V_m is dependent on the contribution of each ionic species. Specifically, the V_m of a cell is determined by i) the permeability of the plasma membrane to different ions and ii) the concentration of these ions on either side of the membrane. The V_m can be expressed by the Goldman-Hodgkin-Katz equation [110]

$$V_m = \frac{RT}{F} \ln \left(\frac{p_K [K^+]_o + p_{Na} [Na^+]_o + p_{Cl} [Cl^-]_i}{p_K [K^+]_i + p_{Na} [Na^+]_i + p_{Cl} [Cl^-]_o} \right) \quad \text{Eq. [1.7]}$$

where p_{ion} is the relative permeability to the ion. The resting potential is in most cell types closest to the E_K due to the high p_K . V_m is also influenced by the E_{Ca} , E_{Na} and E_{Cl} and the currents through anion and cation channels [110,124–127]. The relative

permeabilities and E_{rev} (as calculated using the Nernst equation) of the three most abundant physiological ions in vascular SMCs are summarised in Table 1.1.

1.8 *Ion channels and transporters in vascular smooth muscle cells*

The resting V_m of SMCs is maintained between -40 mV to -60 mV [60,89,97,115,116,122,126,128–133]. This is set by the balance of the hyperpolarising and depolarising currents mediated by the channels present in the cell plasma membrane.

The fact that V_m is more negative than the E_{rev} of all ions apart from K^+ suggests that K^+ currents play a key role in setting the V_m in vascular SMCs. However, as the resting V_m deviates from E_K , other conductances associated with a more depolarised E_{rev} must also be active at rest. The E_{Cl} is the E_{rev} closest to the resting V_m ; despite this, it is unlikely that CaCCs are contributing to the resting V_m as they require activation by increased $[Ca^{2+}]_c$ above resting levels before opening. In accord with this idea are unpublished data from the Tammaro lab that demonstrate that CaCC blockers such as DIDS or Ani9 have no effect on the tension of isolated arteries. In contrast, these agents provoked relaxation of vessel precontracted with agonists that raise $[Ca^{2+}]_c$, such as phenylephrine. The presence of non-selective cation channels such as various TRP channels [69,70,134,135] in smooth muscle may explain why the resting V_m is above the E_K .

In vascular SMCs there are four main types of channels: Ca^{2+} -, Cl^- -, K^+ - and non-selective cation channels. Na^+ channels are also present in some arteries at varying levels but their role in controlling artery tone is debated [136,137].

In the following section, I will provide an overview of the hyperpolarising (K^+) and depolarising (Ca^{2+} , Na^+ and Cl^-) channels expressed in vascular SMCs and ECs, their roles in the control of the V_m and their impact on contraction and blood vessel tone.

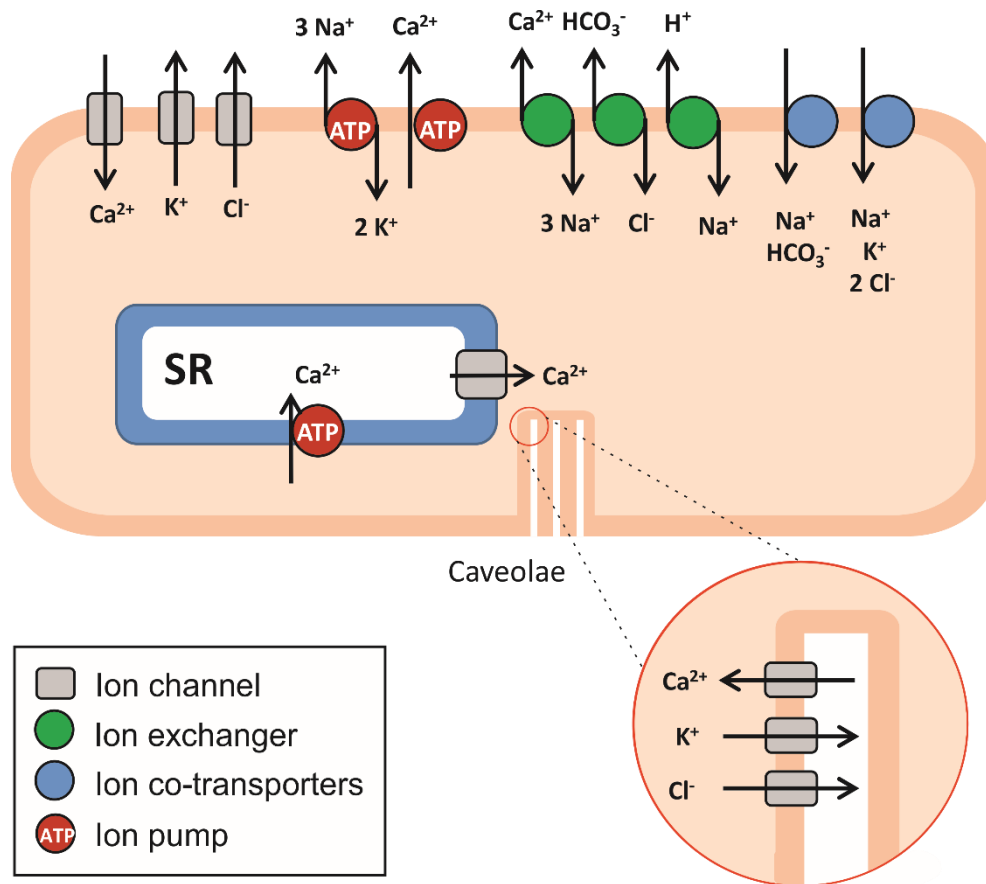


Figure 1.6. Overview of the main types of ion channels, exchangers, transporters and pumps in the SMC.

For clarity, only three (enlarged) caveolae are included in the diagram, one of which is shown in greater detail in the red circle. Caveolae contain an abundance of different channels and transporters but only the channels involved in spontaneous transient outward currents, spontaneous transient inward currents and store-operated Ca^{2+} entry are emphasised in this diagram. Adapted from [3].

1.8.1 K^+ channels

Vascular SMCs express several types of K^+ channel with varying characteristics and roles. These include voltage-gated (K_v), constitutively active (“leak”) two-pore (K_2P) channels [138,139], large-conductance Ca^{2+} -activated (BK_{Ca}), ATP-sensitive (K_{ATP}) and inwardly rectifying (K_{ir}) K^+ channels [140].

K_v channels

The contractile state of SMCs is influenced by the balance of depolarising and hyperpolarising currents leading to either closing or opening of LVGCCs. A key component of the hyperpolarising current is mediated by voltage-dependent potassium (K_v) channels. K_v channels are an important class of K^+ channel found in virtually all smooth muscle cell types [141–148]. These channels remain inactive until a threshold membrane potential is exceeded at which point they mediate an outward, hyperpolarising current [149]. In SMCs the threshold potential is very close to the resting V_m of the cell with an activation range of -55 to -30 mV [140].

The K_v channel family contains 12 classes of pore forming α -subunits composed of 6 TMDs [150]. Within TMD4 there are a series of arginine residues which act as the voltage sensor. In addition to the pore forming unit there are a number of regulatory β -subunits [150]. In vascular SMCs $K_v1.x$, $K_v2.1$, K_v9 , $K_v3.1$, $K_v4.x$ and $K_v7.x$ channels have been identified (see [151] for a comprehensive review). Within the K_v1 and K_v7 there are further subtypes, for example, $K_v1.1$, 1.2 , 1.3 , 1.5 and 1.6 . The K_v channels can be categorised by their inactivation characteristics: 1) rapidly inactivating (e.g. $K_v1.4$) and 2) slowly inactivating (including $K_v1.1-1.3$, $1.5-1.8$ and $K_v7.1-7.5$) [149]. While the slowly inactivating type is expressed in most SMC types, the rapidly inactivating one is expressed only in some locations such

as the aorta and portal vein [140,152]. The wide variety of the different subunits (both α and β) results in heterogeneity in the current amplitude and activation range [148]. Additionally, the varying expression profiles of K_v variants in different arteries can lead to variation in the response to stimuli (e.g. in coronary arteries [153] and reviewed in [125]). It has also been suggested that the activity and localisation of the mitochondria in different SMC types alter the K_v channel sensitivity to voltage [154].

The outward current mediated by K_v channels polarises the plasma membrane leading to closure of LVGCCs and reduces contraction due to the resultant removal of Ca^{2+} influx. Vascular K_v channels have been implicated in the control of the resting V_m and the myogenic response [143,155,156], in addition to certain subtypes ($K_v1.3$) being involved in SMC proliferation [140,157]. By regulating the artery tone and diameter, K_v channels play a key role in controlling regional and global blood flow (reviewed in [147]). In SMCs, the primary K_v channels expressed are $K_v1.x$ and $K_v7.x$. Both $K_v1.x$ and $K_v7.x$ are important contributors to the resting V_m of the SMCs as is evidenced by an increase in the artery tone and a depolarisation when applying K_v channel blockers [144]. This has made them an important potential drug target as activating them could alleviate hypertension. $K_v7.x$ channel have also been strongly implicated as key components in both contractile and dilatory signalling pathways (see [153]). For example, application of ET-1 led to PKC induced inactivation of K_v7 currents, leading to increased arterial contraction as a consequence of reduced basal hyperpolarising currents [158]. K_v7 channels also act as a hyperpolarising and dilatory mechanisms as part of the myogenic effect [141,159]. $K_v1.2$, 1.5 and 1.6 have also been shown to contribute to the negative feedback mechanisms required for the myogenic response.

K_{Ca} channels

Ca²⁺-activated K⁺ (K_{Ca}) channels expressed in SMCs are sensitive to both [Ca²⁺]_c and V_m. While modulated by V_m, the binding of Ca²⁺ to the channel shifts the current voltage relationship to more negative values, resulting in activation of the channel physiological resting V_m values [160]. K_{Ca} channels are classified according to their single-channel conductances. The large conductance K_{Ca} channel (BK_{Ca}) is highly expressed in vascular SMCs [161]. The role of these channels is to provide a negative feedback mechanism to the contraction inducing elevation in [Ca²⁺]_c. Through protein labelling and imaging it was found that BK_{Ca} channels are positioned in close proximity to RyRs [162] and IP₃Rs [163], allowing a direct link between SR Ca²⁺ release and activation of the BK_{Ca} channels [49]. The sensitivity of the BK_{Ca} channels to increases in [Ca²⁺]_c following activation of Ca²⁺ permeable stretch-activated channels (SACs) allows BK_{Ca} channels to play a role in maintaining the myogenic tone [140]. While important for setting the myogenic tone of small vessels [164], the BK_{Ca} channels do not appear to play a significant role in setting the myogenic tone of the microcirculation [165]. This is not due to a lack of BK_{Ca} expression in these tissues as BK_{Ca} currents can still be readily activated by the application of NA [44]. The lack of spontaneous transient outward currents (STOCs) induced by the Ca²⁺ sparks in the microcirculation has been suggested to be due to a decreased sensitivity of the BK_{Ca} channels to Ca²⁺ (requiring <3 µM at V_m of -60 to -30 mV [44]).

In addition to the BK_{Ca} there is also the small (SK_{Ca}) and intermediate (IK_{Ca}) conductance Ca²⁺ activated K⁺ channel. Within the vascular SMCs, IK_{Ca} has a role in proliferation and angiogenesis [166]. SK_{Ca} and IK_{Ca} contribute to the regulation of

vascular tone but through the modulation of the endothelial cells lining the blood vessel lumen [167,168]. In the cerebrovascular circulation, inhibition of IK_{Ca} channels abolished EDHF induced relaxation [169].

K_{ir} and K_{ATP} channels

K_{ATP} and inwardly rectifying K⁺ channels (K_{ir}) belong to the same family of inwardly rectifying K⁺ channels [170].

K_{ir} channels contribute to the resting V_m and thus to the resting vessel tone of resistance arteries. Due to the inward rectification (due to voltage dependent block of the channel by intracellular Mg^{2+} [170]) there is, under normal physiological circumstances, only small outward currents flow through K_{ir} channels. SMC K_{ir} channels have been suggested to be involved in inducing relaxation as part of a EDHF mechanism, as discussed in Section 1.6.2.

In ECs, K_{ir} channels amplify the hyperpolarisation stemming from the activation of the K_{Ca} channels and facilitate propagation of the hyperpolarisation across ECs via gap junctions [171].

K_{ATP} channels are ligand-gated channels [171]. The K_{ATP} channels are inhibited by intracellular ATP binding and thus only mediate a hyperpolarising current at low $[ATP]_i$ [140]; thus, the K_{ATP} channel serves as a molecular sensor that translates the metabolic state into an alteration in V_m [172]. However, the K_{ATP} variant primarily expressed in SMCs (consisting of inwardly rectifying Kir6.1 subunits and ATP-binding cassette SUR2B subunits [173]) is insensitive to ATP [174]. K_{ATP} channels are also regulated by factors other than ATP. These include UDP, GPD and ADP [165], that activate the K_{ATP} channel [174]. The role of the K_{ATP} and K_{ir} channels is

to provide a hyperpolarising current, leading to relaxation of contracted blood vessel and maintenance of the resting V_m (and thus basal blood vessel tone) [165,171,175]. The K_{ATP} channels are inhibited by vasoconstrictors such as Ang and ET-1, as reviewed in [176], which explains one of the underlying mechanisms of the Ang and ET-1 induced constriction.

K₂P channels

K_2P channels, play an important role in many cellular process including cell excitability, cell growth and cell death (reviewed in [139]). Different members of the K_2P channels family are expressed in different tissues and respond to varying stimuli [177]. Additionally, the mechanisms of modulation also vary between the different members of the K_2P family. The majority of the K_2P channels are weakly (or not at all) rectifying and non-inactivating [178]. K_2P channels have two ion permeation pathways and are highly selective for K^+ (due to the TVGYG filter) [179]. Their main function is thought to be setting the resting V_m [180,181]. K^+ efflux via K_2P channels provides a hyperpolarising influence that leads to vessel relaxation. However, in recent years more nuance has been uncovered with the different members being activated by G-proteins, cyclic nucleotides, stretch and acid [180–182]. The stretch activation of these channels allow them to play a part in the myogenic response, provoking vasodilation through the mediation of hyperpolarising K^+ currents [181].

1.8.2 Non-selective cation channels

Non-selective cation channels are ion channel through which there is little selectivity between different positively charged ions. The non-selective cation channels present in vascular SMCs are ligand-gated, stretch-activated, receptor-operated

and SR depletion-activated channels [125]. The permeability of these channels to Na^+ and Ca^{2+} (in addition to K^+) result in a net depolarising current, which leads to activation of the VGCC, Ca^{2+} influx and contraction of the vascular SMCs. Due to their non-selective nature, there is also the possibility that Ca^{2+} entry directly through these channels contributes directly to the activation of the Ca^{2+} dependent contractile machinery.

Receptor-operated channels

Receptor-operated channels (ROCs) expressed in SMCs allow the coupling of innervation and agonist dependent contraction [125,183]. These channels are activated by the intracellular signalling downstream of GPCR activation (e.g. due to diacylglycerol (DAG) production downstream of NA binding to $\alpha 1$ -receptors) [3]. A large proportion of the current mediated by ROCs (such TRPC) is carried by Ca^{2+} and thus these channels contribute both to the activation of LVGCCs (via membrane depolarisation) and raising of $[\text{Ca}^{2+}]_c$ directly [69,184–186]. In addition to the channels activated by the signalling molecules downstream of GPCR activation, there are also ligand-gated channels such as P2X that are activated by extracellular ATP binding [184,187,188]. P2X channels mediate depolarising currents, of which ~10% is carried by Ca^{2+} . ROCs are activated independently of SR Ca^{2+} store release [125].

Store-operated cation channels

Prolonged ROC currents lead to emptying of intracellular Ca^{2+} stores. Once this occurs, intracellularly released agonists activate PM bound Ca^{2+} permeable non-selective cation channels termed store operated cation channels (SOCCs) [189].

The primary role of the SOCCs is to provide a route to refill an emptied SR with Ca^{2+} during extended agonist-induced contraction by allowing an increased influx of Ca^{2+} from the extracellular environment into the cytosol [190]. Activation of SOCCs aids the contraction by increasing the Ca^{2+} influx into the cell. The mechanism linking the depletion of the SR to channel activation was unclear for some time with evidence for multiple theories [125]. The main route of activation appears to be dependent on the unbinding of calmodulin from phospholipase A2 (PLA2) following a decrease in $[\text{Ca}^{2+}]$ in the SR [189]. This process is initiated by the capacitative/ Ca^{2+} influx factor (CIF), the identity of which is yet to be discovered [189]. The channels responsible for store-operated Ca^{2+} entry (SOCE) are primarily members of the transient receptor channel (TRP) family such as TRPC, and Orai1 [135,191–195]. These channels are co-localised with stromal interaction molecules (STIM) on the SR which relays the depleted state of the SR to the PM bound channels which then open to allow Ca^{2+} influx from the extracellular space [47]. The $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCE) has also been suggested to play a role in SOCE as it has the potential to work in the reverse mode (i.e. pumping Ca^{2+} into the cells through the extrusion of Na^+) when the SMC is depolarised [189]. SOCE has also been referred to as ‘capacitative Ca^{2+} entry’. The study of SOCC currents has been hampered by their small amplitude of the current compared to ROC currents that are often concurrently active [190].

Stretch-activated channels

Blood vessels, in particular arteries, experience changes in pressure and thus in the mechanical force on the arterial wall [3]. The force exerted on the artery alters the myogenic tone (see Section 1.6.2). This response is brought about in the activation

of stretch-activated channels (SACs) that lead to an increase in the basal depolarising current and thus a change of V_m [165,196–198]. SACs such as K_2P and TRP channels are also linked to the myogenic response of resistance arteries [181,196].

1.8.3 Ca^{2+} channels

Ca^{2+} enters the cytosol either from the extracellular environment through LVGCC, non-selective cation channels and SOCs, or from intracellular stores such as the SR through IP_3R and RyR channels [49,199,200].

As described in Section 1.5, depolarisation dependent activation of LVGCCs initiates the excitation-contraction coupling pathway in vascular SMCs [126]. SMCs also express transient-opening (T-type) voltage gated channels (TVGCCs) [201,202]. These channels are activated at more hyperpolarised V_m but open with slower kinetics than LVGCCs. TVGCCs are thought to contribute to the myogenic tone of arteries at low pressure [202]. However, it has also been suggested that TVGCCs co-localise with BK_{Ca} and thus contribute to repolarisation [203].

In addition to the plasmalemmal Ca^{2+} channels, there are also channels present in the SR membrane. Activation of these channels leads to movement of Ca^{2+} from the intracellular Ca^{2+} store into the cytoplasm, which contributes to the increase in $[Ca^{2+}]_c$ leading to contraction. The main Ca^{2+} channels present (RyR and IP_3R) are in the SR. Of the RyRs, RyR2 and RyR3 are the isoforms primarily expressed in vascular SMCs. IP_3R and RyR exist in clusters on the SR [204–209]. Compared to LVGCCs, the RyR and IP_3R have larger conductances (~100 pS compared to ~10–20 pS with Ca^{2+} as the primary charge carrier (50–110 mM $[Ca^{2+}]_e$, ~0.1 μM free

[Ca²⁺]_i) as measured with cell-attached patch-clamp or with the lipid bilayer method [210–213]); thus, their contribution to regulating [Ca²⁺]_c is significant [214].

1.8.4 Na⁺ channels

Na⁺ influx in SMCs may occur through non-selective cation channels. There is limited evidence that tetrodotoxin-sensitive voltage-gated Na⁺ (Nav) channels are present in smooth muscle. Nav currents have been described in rat azygous vein and in the rabbit pulmonary artery [215,216], where they were suggested to play a role in the generation of spontaneous activity. However, Nav currents are not ubiquitous in SMCs.

1.8.5 Cl⁻ channels in vascular smooth muscle cells

In vascular SMCs Cl⁻ is accumulated in the cytoplasm through the action of the Na⁺-K⁺-2Cl⁻ cotransporter (NKCC1) [121] and the Cl⁻/HCO₃ exchanger (EA2) [217]. The elevated intracellular Cl⁻ concentration ([Cl⁻]_i) results in an E_{Cl} of ~-25 mV. Thus, opening of Cl⁻ channels will give rise to an inward depolarising current, LVGCC activation and activation of the contractile machinery. There are multiple Cl⁻ channels present in the SMCs including cystic fibrosis transmembrane conductance regulator (CFTR), Ca²⁺-activated Cl⁻ channels (CaCCs), volume-regulated anion channels (VRACs), and the voltage-gated Cl⁻ channels (ClCs) [165,218,219].

CFTR is activated by phosphorylation by the cAMP dependent PKA. Its function in regulating SMC function remains unclear [219], but it has been suggested that the CFTRs are only active at depolarised V_m (above -25 mV). At these potentials, CFTR would mediate an influx of Cl⁻ and oppose further depolarisation and contraction [218].

The CIC family of Cl^- channels are regulated by V_m and (in part) cell volume. The most abundantly expressed member of the CIC family of channels in vascular SMCs, CIC-3, mediates an outwardly rectifying Cl^- current [220]. Importantly, CIC-3 is also a Cl^-/H^+ antiporter that conducts in acidic pH [221]. The primary function of CIC-3 is still under debate; however, its predominant intracellular localisation is suggestive of it having a larger role in acidifying intracellular organelles [219].

Volume-regulated anion channels (VRAC) are weakly Cl^- selective channels that are activated by swelling of the cell [222,223]. Activation following swelling results in Cl^- efflux, thus lowering the osmotic gradient for water entry into the cell, compensating for the swelling and reducing the chance of bursting. VRAC expression has been shown to increase in proliferating SMCs under hypertensive conditions [11]. The increased VRAC expression could explain the more depolarised resting V_m of actively proliferating SMCs [119,224].

Ca^{2+} -activated Cl^- channels (CaCCs) are one of the main depolarising mechanisms in VSCMs. These channels, are activated by both Ca^{2+} and V_m [225]. These channels are considered in further detail below.

The role of CaCCs in agonist-induced contraction

The role of CaCCs such as TMEM16A in vascular SMCs is as a depolarising force which is activated by contractile agonists [225–227]. As described above, the CaCCs serve as mediators for a depolarising efflux of Cl^- from the cell, downstream of IP_3 signalling. This leads to activation of LVGCC and SMC contraction. The increased $[\text{Ca}^{2+}]_c$ due to LVGCC mediated currents maintain the open state of the CaCCs and thus depolarisation [228]. The current voltage relationship for the CaCCs exhibit an outward rectification (as the open probability increases with

membrane depolarisation) [229,230]. However, as the E_{Cl} is ~ -25 mV, the CaCC mediated current prevents excessive (above -25 mV) membrane depolarisation. CaCCs can also be activation by Ca^{2+} sparks, leading to spontaneous transient inward (depolarising) currents (STICs) [231], translating to changes in SMC tone [227]. There is evidence for a CaCC contribution to the myogenic tone [232].

CaCC inhibitors have historically had low potency and selectivity, acting off-target on VRACs, CICs and CFTR [219,227,233–238]. Recently developed compounds such as N-((4-methoxy)-2-naphthyl)-5-nitroanthranilic (MONNA) and Ani9 show much increased efficacy and much improved selectivity, acting solely on CaCCs (TMEM16A and TMEM16B for MONNA and TMEM16A for Ani9 [239,240]).

1.9 **Regulation of intracellular Ca^{2+} in vascular SMCs**

1.9.1 *Ca^{2+} entry and re-uptake*

Cytosolic calcium ($[Ca^{2+}]_c$) is the main determinant of vascular SMC contraction and it is highly regulated. At rest, $[Ca^{2+}]_c$ is low (~ 100 nM) compared to both the extracellular environment and intracellular stores [1,241,242]. This generates an electrochemical gradient for Ca^{2+} entering the cytoplasm. The main routes of Ca^{2+} entry into the cytoplasm are from intracellular stores via IP_3R and RyR , and from the extracellular space through plasmalemmal ROCs, SOCs, SACs and LVGCCs as outlined in Figure 1.7. $[Ca^{2+}]_c$ is lowered through the activity of Ca^{2+} pumps and transporters (Na^+ - Ca^{2+} exchanger), which causes SMC relaxation.

One of the many factors regulating the contractile state of SMCs are neurotransmitters released from synaptic termini. One of these neurotransmitters is

NA, which binds and activates α -adrenoceptors. These G_q -PCRs activate phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into DAG and IP_3 [5,243]. IP_3 activates the IP_3R located on the SR, leading to a release of Ca^{2+} from this intracellular Ca^{2+} store. IP_3 is rapidly degraded by phosphatases (~30 ms half-life) [244]. As such, the diffusion distance to the IP_3R must be minimised; this is through the presence of caveolae as previously described. The rapid degradation of IP_3 also prevents off-target signalling in neighbouring regions [245]. The IP_3R mediated increase in $[Ca^{2+}]_c$ causes opening of Ca^{2+} activated Cl^- channels (CaCC). When activated, CaCCs mediate a Cl^- efflux, leading to membrane depolarisation and opening of LVGCCs [227,246,247]. As the resting V_m of the cell is close to the range of V_m where LVGCCs are activated but not inactivated (window current range), a small depolarisation is sufficient to have a dramatic activating effect on the LVGCCs. The resultant Ca^{2+} influx and elevation of $[Ca^{2+}]_c$ trigger SMC contraction [126].

DAG activates protein kinase C (PKC), which modulates SMC contraction by causing depletion of intracellular Ca^{2+} stores and triggering Ca^{2+} influx across the membrane through SOCCs [189,248]. The IP_3 signalling pathway is also activated when ATII and ET-1 bind their respective GPCRs [82,249].

Once the contractile stimulus ceases, $[Ca^{2+}]_c$ decreases due to the activity of PMCA pump, NCE and SERCA. These proteins expend energy in the form of ATP or utilise the Na^+ gradient to drive Ca^{2+} transport against its electrochemical gradient from the cytosol into the extracellular environment or into intracellular stores [250,251].

Localised Ca^{2+} changes

Compartmentalisation of Ca^{2+} allows selective activation of some Ca^{2+} -sensitive pathways and mechanisms by local changes in $[\text{Ca}^{2+}]$ without activating other Ca^{2+} sensitive pathways. This is a result of the slow diffusion of Ca^{2+} in the cytosol as well as the separation by the membranes of subcellular organelles. The organisation of the organelles is such that the diffusion of Ca^{2+} across the cytosol is decreased through a physical blockage. This compartmentalisation allows $[\text{Ca}^{2+}]_c$ to vary significantly between different micro-milieus within the same cell [47].

Ca^{2+} sparks

Ca^{2+} spark is the term given to localised changes in $[\text{Ca}^{2+}]$ due to release of Ca^{2+} from intracellular stores (i.e. the SR) by RyR and IP_3R [2]. In the immediate proximity of the release site the $[\text{Ca}^{2+}]$ can reach 10s of μM (as extrapolated from electrophysiological and imaging experiments) [252,253]. The close proximity of the SR to the PM means that these events can affect membrane-bound channels (e.g. CaCCs and K_{Ca}) [128,140,227,237]. The co-localisation of these channels with the SR allows Ca^{2+} sparks to initiate either hyperpolarising or depolarising currents mediated by K_{Ca} (spontaneous transient outward currents (STOCs)) or CaCCs (spontaneous transient inward currents (STICs)), respectively [218,254].

Ca^{2+} sparklets

In addition to Ca^{2+} sparks, there are smaller Ca^{2+} events called sparklets. These are short lived increases in the local $[\text{Ca}^{2+}]$ caused by transient activation of clusters of Ca^{2+} permeable channels [47]. The activation of depolarising channels in the proximity of LVGCCs can result in a local depolarisation which in turn leads to LVGCC activation. This depolarisation may not be sufficient to cause a global depolarisation of the cell and thus may not lead to contraction of the SMC. The

frequency of the Ca^{2+} sparklets mediated by the LVGCCs appear to be correlated with the interaction with other membrane-bound proteins such as the scaffolding protein AKAP150 [255]. Ca^{2+} permeable TRP channels (e.g. TRPV4 [256]) are another source of Ca^{2+} sparklets. The Ca^{2+} influx through TRP and LVGCCs can lead to activation of Ca^{2+} -sensitive IP_3R and RyR channels on the SR, which leads to Ca^{2+} -induced Ca^{2+} release (CICR) from intracellular stores [49,165,257]. Both Ca^{2+} sparks and sparklets do not cause SMC contraction as the changes in the local $[\text{Ca}^{2+}]$ is not sufficient to cause a significant enough change in the $[\text{Ca}^{2+}]_c$ for activation of the contractile machinery [47].

Ca^{2+} waves

A third type of transient Ca^{2+} event is the Ca^{2+} wave. This is characterised by an increase in the local $[\text{Ca}^{2+}]_c$ next to the SR. This Ca^{2+} activates neighbouring IP_3R and RyR channels leading to the Ca^{2+} event propagating along the length of the SR membrane and thus across the cell [47,49]. The contribution of IP_3R and RyR to Ca^{2+} waves appear to vary between different artery types [47]. Additionally, the role of Ca^{2+} waves in regulating the vessel tone differs depending on the primary source of the Ca^{2+} for the Ca^{2+} wave [47]. IP_3R -mediated Ca^{2+} waves exert a contractile influence whereas RyR -dependent Ca^{2+} waves caused vasodilation through the activation of K_{Ca} channels [47]. The difference in the response could be due to co-localisation with different channels in the different artery types.

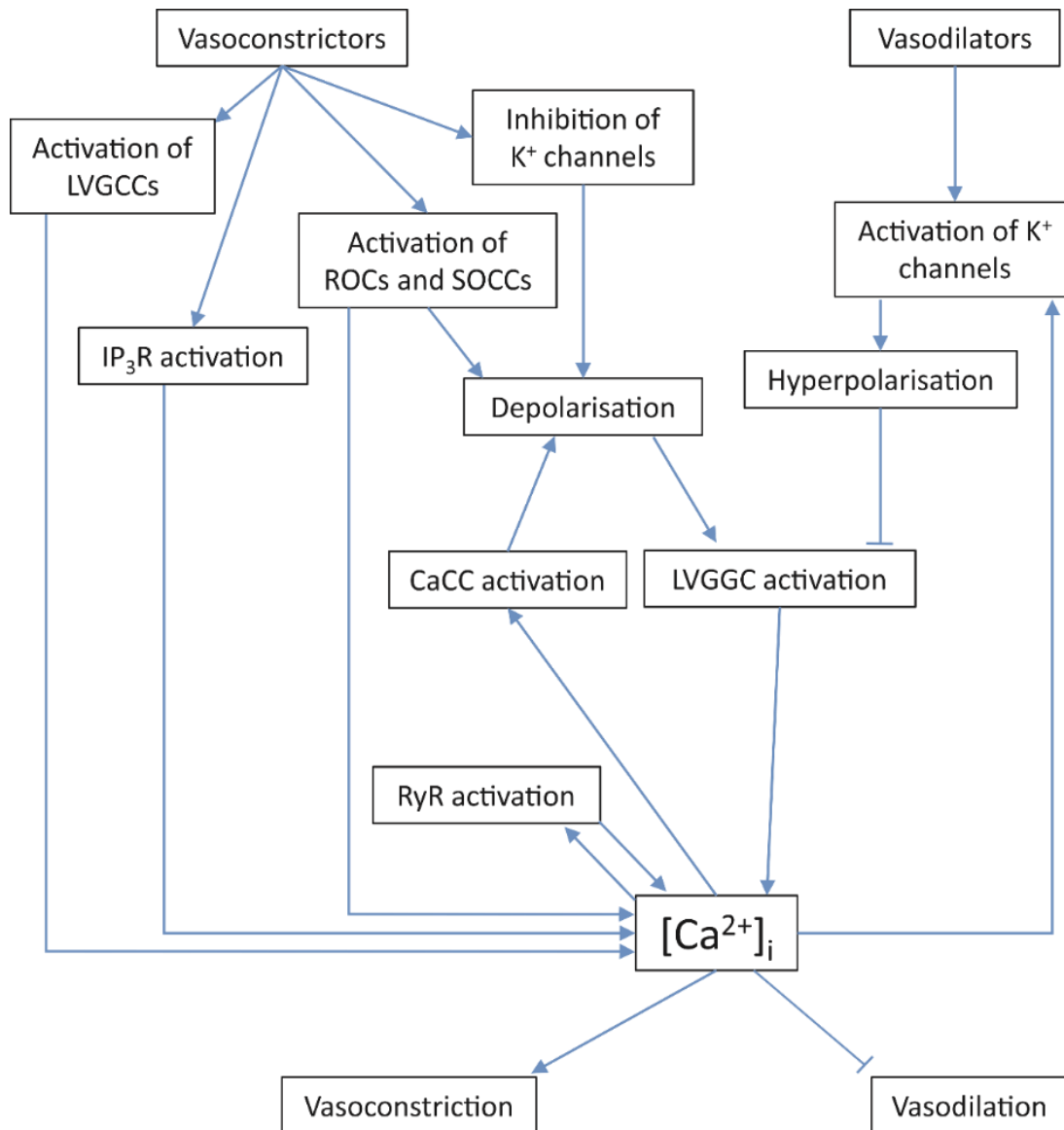


Figure 1.7. Summary of the relationships between ion channels and $[Ca^{2+}]_i$.

The $[Ca^{2+}]_i$ is regulated by a number of ion channels that are activated by vasoactive substances. For clarity, Ca^{2+} transporters that play a significant role in Ca^{2+} handling in the vascular SMCs have been omitted from this diagram. Adapted from [480].

1.10 *Optogenetics including Channelrhodopsin-2*

In the previous sections, I have described the endogenous channels that control the function of SMCs. In this section, I will introduce the idea of utilising exogenous or synthetic channels to control the function of living cells through the application of light (a technology known as optogenetics). Specifically, in optogenetics a combined use of light in conjunction with microbial opsins (light-sensitive proteins) enable control of cellular properties such as cell excitability [258–261]. One of the most frequently used opsins is the blue light activated non-selective cation channel channelrhodopsin-2 (ChR2).

ChR2 is a light-gated cation channel that was first identified in motile green algae where they function as the primary photoreceptors. In these algae, ChR2 is expressed in the eyespot, a specialised organelle that detects light, where they give rise to a fast inward photocurrent. The electrical signal then triggers the activation of action potentials, which are propagated to the flagella to tune their movement and beating frequency.

Heterologous expression of ChR2 in mammalian cells has enabled control neuronal activity [262–265] as well as myocardial excitability and inotropy (see, for example [260,262,266–269]) and skeletal muscle contraction (see [270–272]). This technique allows for control of cell excitability with exceptional spatio-temporal accuracy in a contact-independent fashion [258–260].

Opsins can also cause hyperpolarisation as is the case for halorhodopsin (HR) and archeorhodopsin [273,274]. There are also opsins that control cellular functions other than the V_m (e.g. signalling pathways and cell motility) [275–289]. In the

sections below, I will cover the functional features of wild-type ChR2 as well as the properties of some mutant ChR2 variants that are used in optogenetics.

1.10.1 Structural and electrophysiological properties of ChR2

ChR2 is formed by two identical subunits organised into a dimer [290]. Each subunit presents a 7-transmembrane domain (TMD) structure (see Fig. 1.8). The crystal structure of ChR2 showed that the pore consists of TMD1-3,7 with residues in TMD2 governing ion selectivity [290–295]. Light sensing relies on the presence of the chromophore retinal, a molecule that changes conformation upon photon absorption. Retinal is covalently linked by lysine 257 on TMD7 in each subunit of the ChR2 channel, forming a retinal Schiff base (RSB) [290,296]. Retinal absorbs photons of certain wavelengths (e.g. 480 nm for ChR2 [291]) leading to isomerisation from all-trans-retinal to 13-cis-retinal [297]. This is the starting point for a series of significant structural changes of the protein backbone starting with the rapid (less than a nanosecond) formation of a "preopen" state. This is followed by a transient deprotonation of the RSB. This leads to small conformational changes in amino acid side chains in the neighbouring region causing the channel to open; closure of the channel occurs once the RSB is reprotonated, allowing isomerisation back to the all-trans state [298]. This photocycle is closed when ChR2 reverts to the closed state as light is removed [299]. This series of event is also associated with the transport of H^+ from cytoplasm to the extracellular environment. The physiological significance of this transport, however, is not fully defined [300–302].

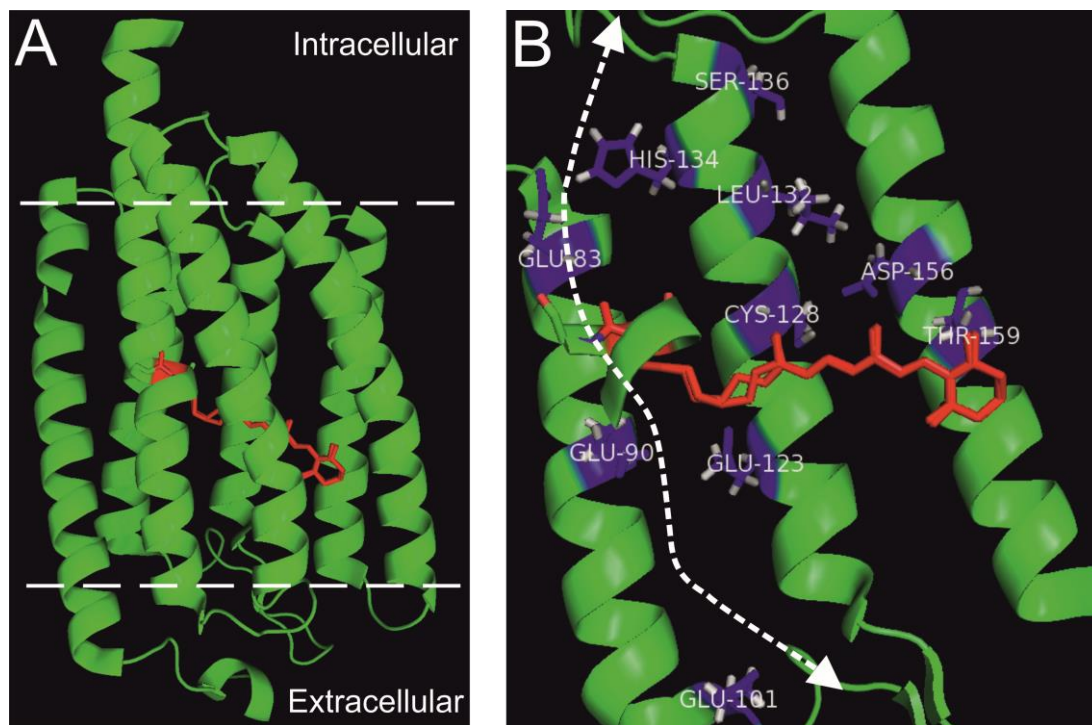


Figure 1.8. ChR2 structure.

A. The structure of one monomer of ChR2. The retinal group is highlighted in red. The estimated positions of the plasma membrane leaflets are indicated by the horizontal white lines. **B.** Residues affecting properties of ion permeation and channel kinetics highlighted in blue. The predicted ion permeation pathway has been highlighted by the dashed white line. Some residues are hidden for added clarity. Figure adapted from [303].

ChR2 is permeant to both monovalent and divalent cations, including H^+ , Na^+ and Ca^{2+} . Thus, the reversal potential for the ChR2 mediated current is ~ 0 mV [260]. ChR2 opening mediates an inward current at physiological resting membrane potentials and its opening typically lead to V_m depolarisation [259,304,305]. The permeability ratio for different ions passing through ChR2 varies depending on the ion size; smaller ions, such as H^+ , have a higher permeability than larger ions, such as Ca^{2+} (e.g. $pH/pNa > 10,000$ estimated using the Goldman-Hodgkin-Katz equation from the E_{rev} of the current with extracellular 115 mM XCl (where X is Na^+ or NMDG), 2 mM $BaCl_2$, 1 mM $MgCl_2$, pH 9, assuming that the cytoplasmic $[K^+]$ was

~100 mM and the pH_i was 7.3 [306]; pCa/pNa : 0.12 with 145 mM NaCl, 3 mM KCl, 4.5 mM NMDG, pH 7.35, and 5 mM NaCl, 3 mM KCl, 80 mM $CaCl_2$, 24.5 mM NMDG, pH 7.35 [306]). The permeability ratio for different ions is obtained by the value of the reversal potential when gradients of two ions are present and compete for the passage through the channel. In this sense the permeability ratio provided a measure of the channels selectivity for various ion types. The ChR2 mediated current is characterised by an early peak that quickly inactivates to a steady-state current level during continuous illumination. This inactivation is due to a combination of different ion permeability of the early and late photocurrents and the existence of multiple open and closed states [299,307,308].

Conductance is a measure of the rate of ionic “throughput” through a channel pore depends on the largest energy barrier(s) the ion has to overcome when passing through the pore. Experimentally, it can be measured from single channel recordings. Alternatively, the slope of the current voltage relation can also provide an indication of the whole cell conductance. ChR2 has a single-channel conductance of 1.1 pS as estimated from reversal potential measurements and based on the assumption that the conductance is Ohmic (the various solutions used for reversal potential measurements are outlined in [306]). This comparatively low to other ion channels such as the BK_{Ca} which has a conductance of ~190 pS (with 144 mM K^+ , 14 mM Na^+ , 2 mM Mg^{2+} , pH 7 on both sides of the membrane and 0.5 μM free intracellular Ca^{2+}) [309] or LVGCCs with a conductance of ~10-20 pS (as measured using patch-clamp electrophysiology with an intracellular solution composed of (in mM) 50 $BaCl_2$, 75 TrisCl, 10 glucose, and 10 HEPES and a bath solution containing 150 KCl, 1 $MgCl_2$, 10 EGTA, and 10 HEPES) [210,211]. However, high expression levels of ChR2 allow consistent depolarisation [259].

1.10.2 ChR2 variants

Various ChR2 mutants have been generated and characterised in the attempt to modify the functional features of ChR2 such as sensitivity to light, sensitivity to different stimulatory wavelengths, ion selectivity, kinetics, and current amplitude. In this section, I will review the electrophysiological features of the principal ChR2 mutants used in optogenetics. The primary sites of these mutations are in the gate or in the retinal binding pocket. In some cases, mutants containing more than a single point mutation have been used to alter more than one electrophysiological feature simultaneously.

Photocurrent amplitude

ChR2 variants with increased photocurrent amplitude have been generated through the substitution of key residues (e.g. threonine-159 (T159) and histidine-134 (H134)). This is a desirable feature as an increased current amplitude allows greater control of the V_m (potentially reducing the level of expression required) [259]. T159 is involved in the transient binding to the retinal during light activation and has a significant effect on the channel properties [310]. Substitution of the threonine with a cysteine (T159C) resulted in a greater binding affinity to the retinal. The T159C mutant exhibit a ~10 fold increase in the peak photocurrent amplitude (potentially due to an increased degree of expression) and an increased sensitivity to blue light stimulation [295,310]. Increasing the binding affinity for retinal also stabilises the protein and result in a reduced degree of protein degradation [310].

The residue at position 134 in ChR2 corresponds to the residue in the proton transporter bacteriorhodopsin which reprotonates the RSB. It was therefore

predicted to play a similar role in ChR2 activation [297,311]. However, it has now been determined that this residue is not the proton donor in ChR2 [291]. Nevertheless, mutation of this residue in ChR2 into an arginine (H134R) led to an increased steady state photocurrent [297]. This was not due to an increase in single-channel conductance but rather a slower off rate [300]. It has been suggested that the more basic arginine may stabilise an intermediate conformational state, favouring the open state by altering the pK_a of the protonated Schiff base [312].

Altered selectivity

The potential uses of an opsin could potentially be broadened by altering the ion selectivity. For example, increasing the anion or K^+ permeability could allow ChR2 to act as a hyperpolarising tool [292,313]. Although it has not been possible to generate a K^+ -selective ChR2 mutant (maximally increased the $pK:pNa$ ratio to ~ 0.6 through the substitution of the serine located in the pore region at position 136 with an alanine (S136A) [314]), progress has been made in the attempt to generate a Cl^- -selective ChR2 mutant [292,315].

One of the key residues involved in altering ChR2 from a cation- to an anion-selective channel is the glutamate at position 90 (E90) [292]. This glutamate residue is positioned at the narrowest point of the pore and is in direct contact with the RSB [292]. Molecular dynamics has shown that the conformational change of the E90 residue following retinal conformational change leads to an opening of the pore [310]. The position of the residue in the permeation pathway means that the charge of the residue has a big impact on the ion selectivity. Mutating E90 to an arginine (E90R) removes the electrostatic repulsion to anions and instead adds an attraction between the anions in the solution and the gate [292]. Similarly, replacing a pair of

glutamates within the permeation pathway (at positions 83 (E83) and 101 (E101), see Fig. 1.8) with glutamine (E83Q) and serine (E101S), respectively, led to increased anion permeability due to reduced electrostatic repulsion between the permeation pathway and anions [292]. This anion selective opsin was termed iChloC and has been used as a hyperpolarising tool to suppress neuronal firing [292,315].

E83 forms a hydrogen bond with H134, across the permeation pathway in the dark state; the conformational change following retinal isomerisation leads to a rotation of the E83 side chain thus allowing access to the pore [316]. Mutating this residue to remove the negative charge had a positive impact on the anion conductance on the E90R mutant described above [315].

ChR2 can also be mutated to exhibit an increased selectivity for specific cations. For example, the T159C substitution led to an increased Na^+ permeability and was thus used as a more potent depolarising tool in neurones [295]. Another example of altered cation selectivity is in a ChR2 variant in which the leucine and position 132 (L132) has been substituted with a cysteine (L132C), which has a six-fold increase in the Ca^{2+} selectivity compared to ChR2 [293]. This mutant has been used to drive action potential trains and it was found that its use led to faster repolarisation due to the activation of K_{Ca} channels by the Ca^{2+} flowing through the mutant channel itself [293].

Altered light sensitivity

One of the main limitations of the majority of the commonly used optogenetic tools is that they are activated at wavelengths that have poor tissue penetrance (see

Section 1.13.1). Increasing the sensitivity of the opsins to the stimulatory light could enable deeper tissue penetration. Similarly, shifting the sensitivity to longer wavelengths (i.e. red-shifted) can also provide deeper penetrance.

Some variants of opsins have been generated to be sensitive to different wavelengths of light. One of these is ReaChR, which is based on a Channelrhodopsin-1 ChR2 chimera with some additional mutations. The key mutation is a substitution of the leucine at position 171 (L171) with an isoleucine (L171I) that extends the life-time of the red-shifted 'transient' states of the photocycle while reducing the degree of desensitisation to these wavelengths of light [317,318]. In combination this leads to a greater sensitivity to red-shifted wavelengths which allows a greater amplitude photocurrent in response to red light [318].

Alteration in kinetics

When substituting residues in ChR2, unwanted changes can also arise. For example, the H134R and T159C substitutions mentioned above (while useful in enhancing the current amplitude and light sensitivity, respectively) slowed the closing and opening kinetics. As a consequence, these opsins are less temporally precise than ChR2 [267,295,319–321].

The glutamate at position 123 (E123) is in close proximity to the retinal [322] (see Fig. 1.8) and has been suggested to be the H^+ acceptor for the RSB [300]. Substitution of glutamate at position 123 with a threonine (E123T) resulted in faster activation kinetics [306,323].

Disrupting the reprotonation of the RSB through the substitution of the aspartate residue at position 156 (D156) with, for example, an alanine (D156A) led to severely delayed channel closure [291]. D156 normally hydrogen bonds with cysteine 128 (C128) on the neighbouring TMD. Substituting C128 to interfere with this hydrogen bond also affects the closing time of the channel, suggesting that this bond is important for the reprotonation process [292,321,324,325].

1.11 Advantages of optogenetic control of cell function

Optogenetics allows tight spatial and temporal control of V_m in a contact-independent fashion, something which direct electrical control does not allow [260]. Further advantages of optogenetics include: 1) the absence of Faradaic reactions: these can be triggered by electrical stimulation using microelectrodes leading to the production of reactive oxygen species (ROS) which may damage the cells and tissue or interfere with ROS signalling pathways [326–335]. For example ROS is involved in ATII induced SMC hypertrophy [329,336], and in the platelet-derived growth factor and ATII induced mitogenesis [330,337]; 2) the possibility of simultaneous stimulation of several cells (electrical stimulation requires one electrode per cell making it impractical for multiple cell stimulation); and 3) optogenetics is less invasive than electrodes which may mechanically damage the tissues when they are inserted [338]. Tissue-specific promoters and viruses carrying opsin RNA allow expression in specific cell types [273,339].

These factors make optogenetics a very useful tool in controlling the V_m of specific cell types at a cellular, tissue and organ level [340].

1.12 Methods for expressing opsins in living cells and tissues

The expression of opsins can be achieved through a number of methods. For example, in model animals, such as mice, expression can be achieved through genetic modification or the use of conditionally active viruses carrying opsin RNA. Cell type-specific promoters allows the targeted expression of opsins in subpopulations of cells in organs [273,341,342].

These different methods have strengths and weaknesses. The generation of genetic mouse models takes a significant amount of time but allows expression of the opsin in all the cells of a particular type through the use of tissue specific Cre recombinase activity. Transgenic mice express the opsin throughout their lifetime. This is in contrast to the viral and cell delivery methods of optogenetically controlling tissue function, which can experience time dependent loss of opsin expression and death of the expressing cells [343,344]. Additionally, the viral and cell-based delivery methods can result in non-uniform expression levels within the tissue [343]. Viral transfer of ChR2(H134R) into murine hearts resulted in an average of 58% of cardiomyocytes expressing the opsin, which allowed the optical pacing of the heart in ~75% of the infected mice [345].

1.13 Challenges to establishing vascular optogenetics

1.13.1 Technical problems associated with uniform illumination

Optogenetics requires stimulatory light to reach the opsins. The blue light typically used is readily absorbed and scattered by tissue and allows penetration only up to a depth of ~1 mm [346,347]. Red cardiovascular tissue readily absorbs the blue

photons, diminishing the penetration further. Thus, the thickness of tissue between the light source and the target cell population has to be minimised. This problem can be bypassed either through stimulating the opsins with different wavelength light or by implanting light sources in the tissue.

The alteration of the sensitivity of optogenetic tools to light of different wavelengths has been a focus over the last decade. As discussed above, opsins that are activated by red-shifted light increased penetration have been generated. These include VChR1 and C1V1 [269,348]. However, these optogenetic tools are not been ideal due to low expression and trafficking problems. Further development has led to the generation of the near infra-red activated ReaChR [318] and Jaws (a hyperpolarising HR mutant). These opsins are activated by light at wavelengths at ~600 nm and show much improved penetration (<10 mm through brain tissue alone or <3 mm into the brain through the mouse skull) [318,349]. Infra-red two photon excitation is another method for deeper penetrating stimulation of ChR2 [348,350]. The use of these opsins can be envisaged to result in a reduction in the level of tissue disruption necessary (i.e. the surgical uncovering of the tissue of interest) for optogenetic stimulation while potentially providing more uniform stimulation throughout the tissue targeted [348,349,351].

Another route of stimulating deeper in tissue is through the surgical implantation of fiberoptics and light emitters. However, the use of rigid light emitting 'optrodes' (combination of electrodes and a light source to stimulate and record electrical activity simultaneously) can be a source of tissue damage [352]. Furthermore, the use of optogenetics *in vivo* outside the field of neuroscience has been hampered by the lack of a stable platform (such as the skull) to attach light sources or optrodes

to. It is possible to minimise the tissue damage associated with surgical implantation through the thinner and more lightweight components [353,354]. Additionally, the development of stretchable light emitting sheets could potentially be adhered to an organ surface (where the stretchable property could allow contraction with minimal impairment) and stimulate opsins *in vivo* [355]. The use of implanted optrodes has allowed the neural optogenetics to be employed in non-anaesthetised animals moving freely [356,357].

Despite these limitations, optogenetics remain a highly valuable tool with many advantages over more traditional experimental techniques.

1.13.2 Native blue light induced relaxation of arteries

One challenge which is particular for establishing vascular optogenetics is that blue light, of similar wavelengths required for opsin activation, causes relaxation in various artery types if applied in the presence of a contractile agonist [358–360]. The mechanism behind this blue light induced relaxation was unknown until 2014, when Sikka et al. identified the light sensitive GPCR melanopsin in SMCs as the key component in this vasodilatory pathway [361]. The mechanism behind the melanopsin mediated response was not fully elucidated but a number of potential routes were excluded, including NO, carbon monoxide, or cytochrome p450-derived vasoactive products (e.g. EEA) signalling, and via PKG activity. The activation of melanopsin did nevertheless lead to SMC hyperpolarisation and relaxation, resulting in increased blood flow in the stimulated tail artery [361]. Knocking out melanopsin in SMCs abolished the blue light induced relaxation. It is suggested that this vasorelaxation could play a role in the circadian regulation of blood flow.

The implication of melanopsin mediated photorelaxation for vascular optogenetics could be significant as any depolarising force mediated by opsin activation would need to overcome the apparent melanopsin mediated hyperpolarising force activated by the same wavelengths of stimulatory light. This is particularly important for any *in vivo* applications of vascular optogenetics as the circulating levels of catecholamines vary depending on the extent of the sympathetic drive. However, as the effect is not observed in the absence of contractile agonists such as NA or phenylephrine (PE), vascular optogenetics could still provide a useful method for the study of isolated arteries or SMCs.

1.14 Optogenetic control of vascular function

Optogenetic control of SMC V_m and function

As described in Section 1.6, SMCs contract in response to contractile agonists such as NA through the activation of the IP_3 signalling pathway which leads to CaCC mediated depolarisation and activation of LVGCCs and thus contraction. Expression of depolarising opsins (e.g. the ChR2 mutant ChR2(H134R)) could bypass the need for contractile agonists signalling, thereby causing contraction in an agonist independent fashion. This could offer the opportunity to control SMC V_m and vessel tone both *ex vivo* and *in vivo*. In isolated blood vessels, contractile agonists can take multiple minutes to elicit a stable response [143,362]. Therefore, the opsins used for vascular optogenetics needs to be able to maintain stable current over an extended stimulation.

In 2015, Wu *et al.* reported a mouse model for vascular optogenetics. Their work included the expression of ChR2(H134R) selectively in SMCs which allowed light-dependent depolarisation and induction of SMC contraction (and thereby arterial contraction) [363]. Their study represents the first application of optogenetics to directly control vascular function. In this study a series of cellular and *ex vivo* tissue and organ experiments were carried out. Isolated aortic SMCs expressing ChR2 demonstrated light-induced currents. The tone of isolated mesenteric artery rings increased in response to high intensity blue light stimuli (ranging from 10 mW/mm² to 24 mW/mm²), evoking maximal ChR2 activation [306,325]. Furthermore, it was possible to optogenetically modulate the perfusion pressure of the coronary and renal circulations in isolated hearts and kidneys [363]. Isolated mesenteric artery rings could maintain contraction for long periods of time (up to 1 hour) in response to constant exposure to blue light. More recently, optogenetics has been used to gain insights into the role of arterioles and pericyte-covered capillaries in the control of cerebral blood flow [364].

Optogenetic control of EC V_m and function

Optogenetics can also be employed in the study of vascular ECs and could be highly useful in the study of the crosstalk between ECs and SMCs. Zhang *et al.* showed that expressing a depolarising opsins such as ChR2(H134R) in ECs enables optogenetic control of the EC V_m [365]. The effect of this on SMC V_m and vascular tone could be investigated using myography and the tandem cell unit approach to electrophysiology [319].

Surprisingly, ChR2(H134R) mediated depolarisation of ECs led to an increase in blood pressure in both renal and coronary arteries, independent of ET-1 or TxA2

signalling [365]. The underlying mechanism was not fully elucidated but it was hypothesised that it was either due to light-dependent depolarisation associated with reduced release of endothelium-derived vasodilators or that it stimulates K^+ efflux from the ECs that in turn causes depolarisation of vascular SMCs [365].

These pioneering studies of optogenetics in SMCs and ECs have demonstrated the utility and potential of optogenetics in vascular research. Critically important mechanistic aspects, however, need to be addressed to enable a complete interpretation of vascular optogenetics experiments and to exploit this technology in the *in vivo* setting. In my studies, I have aimed to define key mechanistic aspects. Key aspects that should be addressed include: (1) whether the arterial responses over an adequately wide range of light stimulations to determine whether different degrees of ChR2 activation enable fine, graded control of vessel tone; 2) if melanopsin-mediated relaxation of pre-constricted wild-type arteries [358–361] could be overcome; (3) the precise ionic mechanisms linking light stimulation with arterial contraction; and 4) whether light stimulation could affect the pH_i of SMCs. The answer to these questions will establish whether the responses to ChR2 activation reflect its depolarising effect as opposed to the alternative possibility that they are secondary to changes in pH_i (due to its H^+ permeability) and $[Ca^{2+}]_c$ (by providing an non-physiological conduit of Ca^{2+} entry [295,304,306,311,366]). The effects on pH_i may be particularly pertinent as pH_i is a key modulator of excitation-contraction coupling and other fundamental cellular functions [367,368].

Potential uses of optogenetics to study vascular function

The potential ways that vascular optogenetics can be applied for the study of vascular function are numerous. For example, optical stimulation can be precisely

targeted to a specific region of blood vessels. This high spatial resolution may facilitate the elucidation of the nature of the neurovascular coupling in the brain. The temporal resolution of this optogenetic approach of inducing vessel constriction allows better control of vascular tone where timing is critical. Additionally, blue light stimulation has the potential of being both more cell type specific and with a faster application time than pharmacological agents. Additionally, controlling blood flow through restriction or relaxation of blood vessels could allow the induction or relief of ischaemia. Optical vasoconstriction can be used to set a basal vascular tone for the study of the vasodilation elicited by potential therapeutic agents without concurrent contractile agonist signalling (otherwise required to induce a degree of contraction). Optical vasoconstriction will be useful to manipulate the metabolic rate temporospatially by reducing O₂ and nutrients supplied to a certain tissue. This could even allow clinical applications if opsins could be safely delivered to a specific tissue. Furthermore, vascular optogenetics should enable the functional coupling between vascular ECs and SMCs to be investigated with unprecedented cellular and spatial specificity.

1.15 Aims and objectives

The main aim of this thesis is to establish optogenetics in the vasculature. While this technique has already been used in a small number of studies [363–365], major mechanistic aspects remain unknown that have been addressed for the validation of this (potentially) useful technology.

The aims of this thesis were to:

- 1) To perform a thorough, side-by-side, comparative study of the characteristics of ChR2 and the commonly used ChR2 variant ChR2(H134R), to define which one of these variants would be the most suitable for vascular optogenetics;
- 2) Generate a mouse model in which ChR2(H134R) was selectively expressed in SMCs and study the effect of blue light on currents, V_m and artery tone;
- 3) Elucidate the precise ionic and cellular mechanisms that couple ChR2(H134R) activation to an increase in arterial tone; and
- 4) Define the biophysical properties of a Cl⁻-selective variant of ChR2 to examine its suitability for future work involving vascular optogenetics.

Chapter 2

Materials and Methods

2.1 ***Chemicals and reagents***

Chemicals were purchased from Merck, UK, unless stated otherwise. Ani9 (synthesised by Dr. Angela Russell's lab, University of Oxford), DIDS, Nifedipine, clotrimazole, 2-APB and MONNA were dissolved in DMSO. Ryanodine was dissolved in water. Primers were purchased from IDT Technologies (Belgium).

2.2 ***Use of mice as a model organism***

All animal care and procedures were approved by the University of Oxford Ethics Committee and were in accordance with UK Home Office guidelines and the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.2.1 *Generation of transgenic mice*

Mice selectively expressing the channelrhodopsins-2 variant ChR2(H134R) in SMCs (ChR2(H134R)-SM mice) or ECs (ChR2(H134R)-EC mice) were generated by crossing pre-existing strains termed GROS, TTCR or RTEK. GROS mice were homozygous for ChR2(H134R) tagged with the enhanced yellow fluorescent protein (eYFP) coding sequence (ChR2(H134R)-eYFP) inserted downstream of a loxP-flanked STOP cassette within the Gt(ROSA)26Sor locus (012569, Jackson Laboratories, USA). TTCR mice were homozygous for the Cre recombinase coding sequence under the control of mouse transgelin (smooth muscle protein 22- α) promoter (004746, Jackson Laboratories, USA). RTEK mice were homozygous for the Cre recombinase coding sequence under the control of endothelium specific receptor tyrosine kinase (Tek) (008863, Jackson Laboratories, USA).

ChR2(H134R)-SM mice were generated by crossing GROS and TTCR mice. ChR2(H134R)-EC mice were generated by crossing GROS and RTEK mice. In the offspring, the removal of loxP-flanked STOP cassette by Cre recombinase led to selective expression of ChR2(H134R)-eYFP in SMCs or ECs for ChR2(H134R)-SM and ChR2(H134R)-EC, respectively. TTCR mice were used as control.

Mice were kept in individually ventilated cages and fed *ad libitum*. Adult mice (8-15 weeks) were culled by cervical dislocation in accordance with UK Home Office guidelines.

2.2.2 DNA extraction for genotyping

DNA was extracted using the phenol/chloroform extraction method [369]. Mouse ear punches (taken at ~14 days after birth) were digested overnight in Eppendorf tubes containing 500 µl cell lysis solution (Qiagen, UK) supplemented with 0.01% w/v proteinase K (Invitrogen, UK) at 55 °C. The next day 500 µl phenol/chloroform was added to each sample. The sample was then vortexed and centrifuged at 13,000 RPM for 5 min at 4°C. The top aqueous layer containing genomic DNA was transferred to a new Eppendorf tube, and 500 µl ice-cold isopropanol was mixed with the samples and centrifuged at 13,000 RPM for 20 min at 4°C. The supernatant was discarded and the DNA pellet was washed with 500 µl 70% ethanol and centrifuged at 13,000 RPM for 5 min at 4°C. The ethanol was removed and the DNA pellet was dried at 37°C for 15 min. DNA was dissolved in 100 µl of double distilled water and incubated at 55°C for 60 min. The concentration of the isolated DNA was ascertained through spectrophotometry using a Nanodrop 2000c spectrophotometer (Thermo Scientific, UK). Nucleic acids that make up both DNA and RNA have a maximal absorption at 260 nm. Thus, the DNA concentration and

purity was determined through the measurement of the optical density (OD). The optical density (OD) of 1 measured at 260 nm wavelength indicates 50 µg/ml of dsDNA [369]. The ratio of OD measured at 260 nm wavelength over that measured at 280 nm or 230 nm wavelength indicates purity of the DNA relative to protein and organic or chaotropic compounds, respectively. 260/230 and 260/280 ratios were considered acceptable when over 1.8.

2.2.3 Genotyping

Genotyping was carried out by performing polymerase chain reaction (PCR) on DNA extracted from TTCR, RTEK and GROS mice (as outlined in Section 2.2.2). PCRs were carried out with primers complementary to the transgene and primers complementary to the regions immediately 5' and 3' of the insertion point. The primers were designed to generate different length PCR fragments depending on the genotype of the mice (see Table 2.1). PCRs on homozygous TTCR, GROS and RTEK mice resulted in only one size band whereas heterozygous mice produced two different sized bands.

Genotyping Chr2(H134R)-SM mice required two reactions to test for the presence of both the Cre recombinase and Chr2(H134R) genes. PCRs to amplify Chr2(H134R) and Cre DNA constructs were performed using a mixture of extracted DNA (1 µl), forward and reverse primers (0.5 µl of each, see Table 2.1), 5 µl HotStar Taq Master Mix containing HotStar Taq DNA Polymerase, 1.5 mM MgCl₂, dNTP mix, PCR buffer (Qiagen, UK), and 3 µl molecular biology grade H₂O with a standard PCR protocol based on an initialising step at 95 °C, a 2 min denaturing step at 95 °C, a 30 s primer annealing step (60-65 °C) and an extension step at 68 °C.

The PCR products were mixed with loading dye and loaded on an agarose gel as described in section 2.2.4.

Table 2.1. Genotyping primers.

	Primer name	Sequence	Band size
Chr2(H134R)-eYFP	Wild type (Forward)	AAG GGA GCT GCA GTG GAG TA	297
	Wild type (Reverse)	CCG AAA ATC TGT GGG AAG TC	
	Mutant (Forward)	ACA TGG TCC TGC TGG AGT TC	212
	Mutant (Reverse)	GGC ATT AAA GCA GCG TAT CC	
TTCR and RTEK Cre Recombinase	Transgene (Forward)	GCG GTC TGG CAG TAA AAA CTA TC	100
	Transgene (Reverse)	GTG AAA CAG CAT TGC TGT CAC TT	
	Internal Positive Control (Forward)	CTA GGC CAC AGA ATT GAA AGA TCT	324
	Internal Positive Control (Reverse)	GTA GGT GGA AAT TCT AGC ATC ATC C	

Oligonucleotides used in genotyping for control, Chr2(H134R)-SM and Chr2(H134R)-EC mice (in 5' to 3' direction). The band size is given in base pair (bp) of the amplicon.

2.2.4 Gel electrophoresis

Gel electrophoresis relies on the electric charge of DNA to separate different length DNA fragments. DNA samples were mixed with 2 µl of 6x loading dye and loaded on a 1%-2% agarose SeaKem agarose (Lonza, UK) gel made using TAE buffer (Fischer Scientific, UK), supplemented with 5 µl/100 ml ethidium bromide (Promega, UK). The DNA bands were visualised following the running of the gel using an ultra-violet light source and a digital camera. The fluorescence signal of ethidium bromide

is ~25 fold brighter upon binding double stranded DNA [370]. In aqueous solutions, ethidium bromide is excited at ~280 nm, and emits at a wavelength of 605 nm [371].

2.3 **Molecular biology**

2.3.1 *Vectors used and site directed mutagenesis*

Site directed mutagenesis of ChR2(H134R) was carried out to generate ChR2 and iChloC using QuikChange Lightning kit (Agilent, UK). This method relies on a mismatch (≤ 3 nucleotides) between a pair of complementary primers and the template DNA to induce the desired mutation. The primers were designed so that the mismatch was at the centre of each primer to ensure high stability between the primer and template DNA. The mutagenesis reaction was performed as detailed in the manufacturer's instructions. In brief, a PCR reaction was performed using a mixture containing 1 μ l extracted DNA, 1.5 μ l forward and reverse primers (see Table 2.2), 1 μ l QuikChange Lightning enzyme, 1 μ l dNTP mix, 1.5 μ l QuikSolution, 5 μ l 10X reaction buffer and H₂O (to a final volume of 50 μ l) with a standard PCR protocol. Following the PCR, 1 μ l of the restriction enzyme Dpn1 was gently mixed into the mixture to digest the DNA template. Dpn1 only cleaves DNA which has been methylated and thus will only cleave the template DNA (isolated from *E. Coli*) and not digest the newly formed plasmids [372].

The samples were then used for the transformation of XL10-Gold ultracompetent *E.coli* were then transformed and inoculated as described in 2.3.2.

Table 2.2. Primers used for site-directed mutagenesis.

Primer name	Sequence (5'–3')
ChR2(E83Q)-F	CAT GCG GCT GGG AGC AGA TCT ATG TGT G
ChR2(E83Q)-R	CAC ACA TAG ATC TGC TCC CAG CCG CAT G
ChR2(E90R)-F	GTG TGC GCC ATT CGA ATG GTT AAG GTG
ChR2(E90R)-R	CAC CTT AAC CAT TCG AAT GGC GCA CAC
ChR2(E101S)-F	CTC GAG TTC TTT TTT TCG TTT AAG AAT CCC TCT
ChR2(E101S)-R	AGA GGG ATT CTT AAA CGA AAA AAA GAA CTC GAG
ChR2(R134H)-F	GTC ATC CTT ATC CAC CTG AGC AAC CTC
ChR2(R134H)-R	GAG GTT GCT CAG GTG GAT AAG GAT GAC
ChR2(D156N)-F	GGA CTC CTT GTC TCA AAC ATC GGG TGT ATC GTG
ChR2(D156N)-R	CAC GAT ACA CCC GAT GTT TGA GAC AAG GAG TCC
ChR2(T159C)-F	TCA GAC ATC GGG TGT ATC GTG TGG GGG
ChR2(T159C)-R	CCC CCA CAC GAT ACA CCC GAT GTC TGA

Oligonucleotides used for site-directed mutagenesis of ChR2 (in 5' to 3' direction).

2.3.2 Transformation and inoculation of chemically competent cells

All expression vectors were amplified in bacteria. ChR2 and ChR2 variants were subcloned into a pcDNA3.1 expression vector which contained an ampicillin resistance cassette to permit the selection of bacteria successfully transformed for DNA amplification. The DH5α strain of competent *Escherichia Coli* (*E. coli*) cells (Invitrogen, UK) were used as they lack pathogenic mechanisms required for infection and were unlikely to survive in host tissue or cause disease [373]. The purified plasmids (2 µl (10 ng total)) was gently mixed with 50 µl DH5α cells then

incubated on ice for 30 min, heat-shocked at 42 °C for 20 s, incubated on ice for another 5 min before 500 µl pre-warmed super optimal with catabolite suppression (SOC) medium was added to the cells. The SOC medium provides essential nutrients necessary for bacterial growth. The cells were then incubated for 1 h at 37 °C in a shaking incubator (225 RPM). Finally, 100 µl of the transformed cells was spread on pre-warmed Luria-Bertani (LB) agarose plates containing 50 µg/ml ampicillin (Merck, UK) and grown for 14-16 h at 37 °C. Only bacteria successfully transformed with the pcDNA3.1 vector (containing the ampicillin resistance cassette) would be resistant to the ampicillin. If no colonies were present on the control plate, colonies were picked and used to inoculate 5 ml or 50 ml LB Broth (Merck) and incubated at 37 °C overnight in a shaking incubator (300 RPM) in preparation for either MiniPrep or MidiPrep (both Qiagen, UK), respectively.

2.3.3 Extraction and purification of plasmid DNA

QiaPrep Spin MiniPrep kit and HiSpeed Plasmid Midi Kit (Qiagen, UK) kits were used to extract and purify the DNA vectors isolated from transformed *E. coli* cells, as detailed in the manufacturer's instructions. This procedure is based on an alkaline lysis of bacterial cells, subsequent neutralisation and DNA recovery developed by Birnboim and Doly [374]. The plasmid DNA is then passed through an ionic exchange column (QIAprep silico membrane) under high salt solution conditions. After a wash the DNA was eluted using a low salt buffer solution (Buffer TE) containing Tris and EDTA to solubilise DNA and to inhibit nuclease activity, respectively. The quantity and quality of the DNA was assessed using a ND-1000 spectrophotometer (NanoDrop) as described in Section 2.2.4. Extracted DNA was sequenced commercially by GATC Biotech (Germany) to verify that the desired mutation was achieved.

2.4 HEK-293T cell culture and transfection

2.4.1 Cell culture

HEK-293T cells are commonly used as an expression system for ion channel studies (e.g. [235,375]). HEK-293T cells were used for the heterologous expression of ChR2, ChR2(H134R) and iChloC. HEK-293T cells efficiently express the protein coded for by plasmids containing SV40 enhancers as this cell line stably expresses the SV40 large T-antigen [376]. The SV40 large T-antigen binds to SV40 enhancers increasing the transcription and thus protein production [376,377]. All vectors used in this work contained SV40 enhancers.

The HEK-293T cells were grown in Dulbecco modified Eagle medium (DMEM) (Gibco, UK), supplemented with 10% (v/v) foetal bovine serum (FBS) (Gibco, UK) and 0.5% (v/v) gentamicin (Merck, UK). This mixture was termed complete medium. HEK-293T cells were cultured in T12 flasks (Fisher Scientific, UK) at 37 °C at 5% CO₂. The complete medium was replaced every 2-3 days and the cells were split at ~80% confluency. In brief, the complete medium was removed from the T12 flasks and the cells were gently washed with DPBS (Gibco, UK) before a 1 min incubation at 37 °C with 0.5 ml trypsin solution (0.5 g/l trypsin, 0.2 g/l EDTA (Merck, UK)). Trypsin activity was quenched through the addition of 3 ml complete medium. The cells were then transferred into a 15 ml test tube and centrifuged at 200 RPM for 3 min after which the supernatant was removed. The cell pellet was resuspended in 2 ml complete medium and a portion of cell suspension was transferred to a new T12 flask and incubated. The number of cells per μ l was established through the use of a haemocytometer before cells were plated in 35x10 mm cell culture dishes (Corning, USA) at a density of 40,000 cells per dish for electrophysiology.

2.4.2 *Heterologous expression in HEK-293T cells*

HEK293T cells were transiently transfected using 1.5 µg vector DNA containing either ChR2, ChR2(H134R) or iChloC using FuGENE 6 transfection reagent (Promega, UK) following manufacturer's instructions (1.5 µl FuGENE6 per µg DNA). The transfection reagent coats the genetic material in lipid. This complex can enter the cells through the fusion with the plasma membrane. The transfection mixture was added to the cell culture dishes ~16 h after the cells were plated, and incubated for 18-24 h to allow expression of the vector.

2.5 *Isolation of native SMCs*

Isolated arteries (aorta, mesenteric and pulmonary artery) were washed in cold PSS (for composition see Section 2.15) and connective tissue was removed. Isolated arteries were cut open longitudinally before being cut into 2 mm long sections and washed with dissociation medium (DM). The composition of DM was (in mM): 110 NaCl, 5 KCl, 10 HEPES, 0.5 KH₂PO₄, 0.5 NaH₂PO₄, 10 NaHCO₃, 10 taurine, 0.5 EDTA, 10 glucose, 0.16 CaCl₂, 2 MgCl₂, 0.03 phenol red (pH 7.3). The artery fragments were then incubated in DM supplemented with 1.5 mg/ml of the protease papain (Merck, UK) and incubated at 4°C for 1 h to allow the papain to thoroughly enter the tissue. The reducing agent dithiothreitol (0.5 mg/ml) was subsequently added to the medium to increase the catalytic activity of papain [378]. The sample was then incubated at 37°C for 5 min. The tissue pieces were then transferred into a new vial containing 0.4 mg/ml of the endopeptidase collagenase (type XI from *Clostridium histolyticum*, Merck, UK) and incubated at 37°C for 4 min. The tissue pieces were subsequently washed three times in fresh DM to remove traces of

active digestive enzymes and gently triturated ~1 min with a smoothed glass Pasteur pipette to dislodge individual SMCs. The isolated SMCs were kept on ice and used within 5 h for electrophysiological or imaging experiments.

2.6 ***Blue-light stimulation systems for optogenetics***

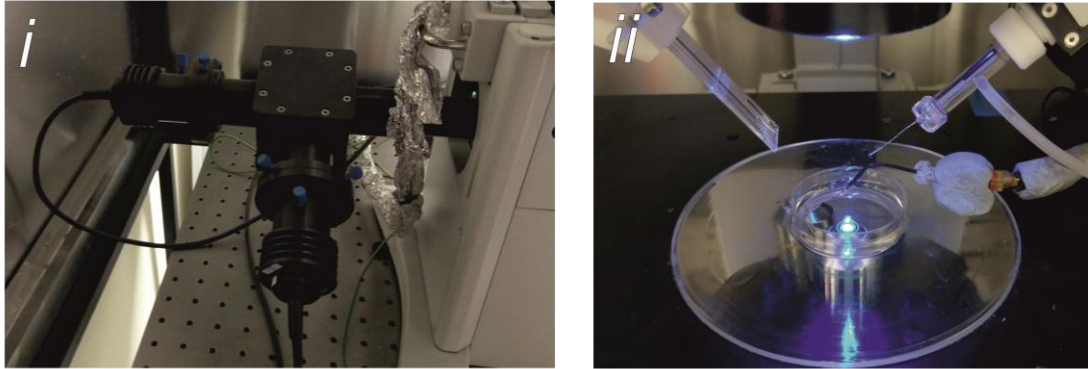
To study the response of isolated cells and tissues to blue light stimulation different LED and light guide systems were required. In patch-clamp experiments, optical stimulation of ChR2(H134R)-expressing SMCs was performed using an optoLED system (Cairn Research, UK), comprising of a 3.5 W blue light (470 nm) emitting diode (LED) mounted on the rear port of an inverted microscope following the manufacturer's instructions (IX51, Olympus, UK). The optoLED system was controlled by GePulse software (Dr M. Pusch, CNR, Italy) with an analogue-to-digital and digital-to-analogue converter (USB-6221, National Instruments, UK).

For myography experiments, blue light was provided by a 5 W LED, controlled by a power supply made in-house and delivered through a glass window positioned at the bottom of the myography bath chambers. Intensity and duration of the stimulus were digitally controlled. In pH imaging experiments, the same LED power supply system was used and blue light was delivered to the sample through a liquid light guide with a core diameter of 3.0 mm (Newport, USA), reaching the sample on the microscope stage perpendicularly from above.

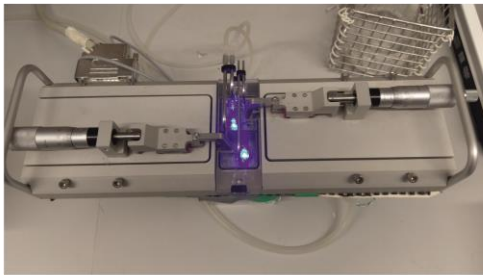
Light intensity (in mW/mm²) was measured at the sample (cell or tissue) site using a Newport digital optical power meter (Model 843, Newport, USA), with a sensor area of 0.4 cm x 0.4 cm, with the measured wavelength set to 470 nm. Calibration

performed at the beginning of the experiments confirmed that the LED current (in the range 0–1.0 A) was linearly related to illumination wattage at the sample plane.

A



B_i



B_{ii}

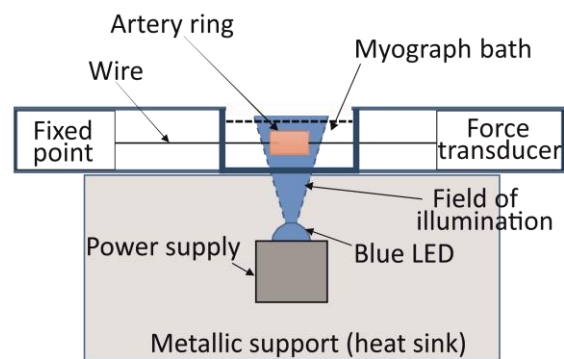


Figure 2.1. Light delivery systems.

A. Patch-clamp electrophysiology set up with light delivery. **i.** Light input at the rear of a IX51 microscope, as described in the manufacturer's instruction. **ii** Field of stimulation for patch-clamp recordings. **B.** Myography set up with light delivery. **i.** Myograph with LEDs shining through the window at the bottom of each well. **ii** Schematic of the myograph LED set up. This side view shows the origin of the light from an LED placed below the window allowing light to enter the bottom of the bath chamber.

2.7 *Electrophysiology*

The patch-clamp technique allows the measurement of the electrical properties of cell membranes [379–383]. For patch-clamp electrophysiology, the cell can be considered part of an electrical circuit. The cell membrane can be described as a capacitor as it is essentially an insulator separating two conductors (solutions with ionic charge carriers). Capacitors are charged when a voltage is applied across the capacitor. The amount of charge stored is dependent on the surface area; therefore, the size of the cell can be estimated based on the capacitance. In the cell, this potential difference across the membrane is established through the uneven distribution of charged particles (e.g. ions). The relationship between capacitance (C) and charge (Q) is given by:

$$C = \frac{Q}{\Delta V} \quad \text{Eq. [2.1]}$$

where ΔV is the change in voltage. The ion channels present in the plasma membrane can be considered as variable resistors (R) that control the ion flux and ionic current (I_I) by opening and closing. Once the channels open, voltage across the membrane decreases allowing the membrane (i.e. the capacitor) to discharge. This discharge generates a capacitive current (I_C) which is dependent on the change in voltage per second ($\Delta V/\Delta t$). The capacitive current is also seen when applying a voltage across the membrane in the voltage clamp configuration of the patch-clamp method. The total current (I_T) in the circuit is a sum of I_I and I_C . However, as can be surmised from the above information, the I_C is only generated when there is a change in voltage. Therefore, the transient I_C is only visible at the

beginning of a voltage step during patch-clamp experiments. After this, the current flowing across the membrane is proportional only to the I_i .

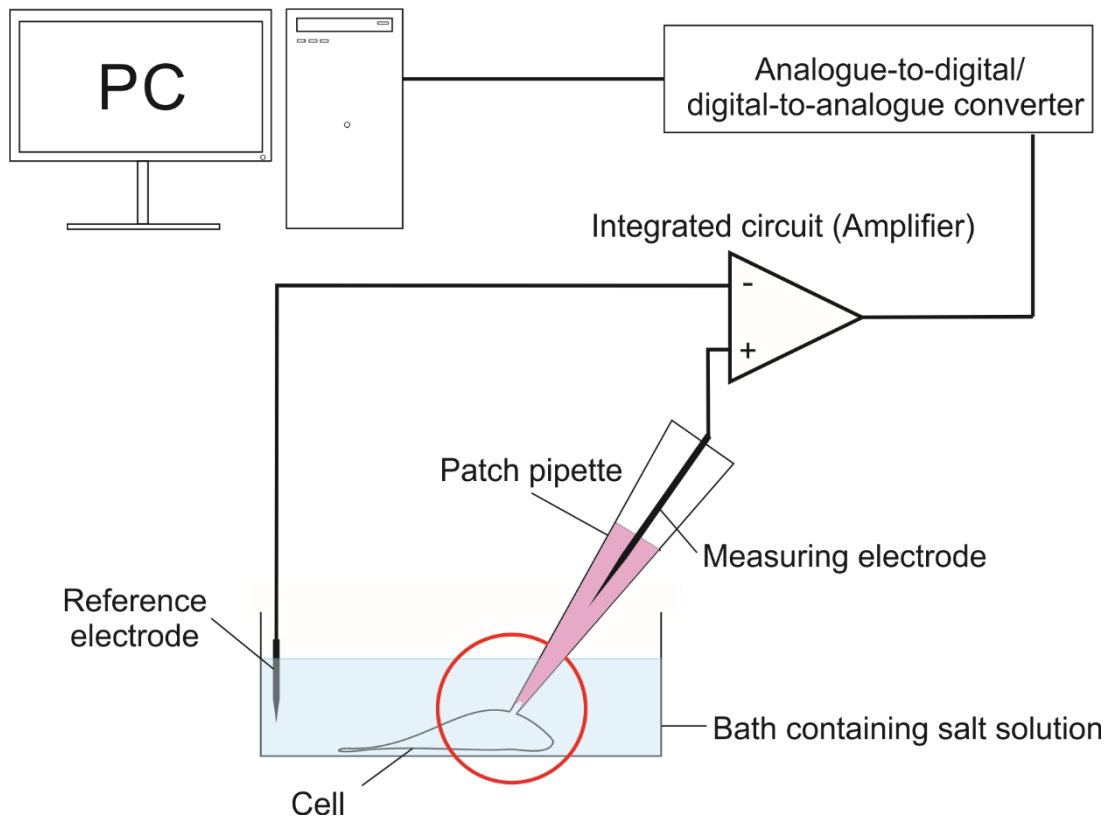


Figure 2.2. Diagrammatic representation of whole-cell patch-clamp set up.

Red circle is expanded in Figure 2.3.

Patch-clamp electrophysiology enables the role of proteins specialised to transport charges across membranes to be studied through a variety of different configurations. Electrical recordings from cells or membrane patches can be achieved through multiple different configurations of the patch-clamp technique, each one particularly suitable for certain aspects of electrophysiology. For the purposes of this study whole-cell and perforated patch configurations were used (see Fig. 2.3).

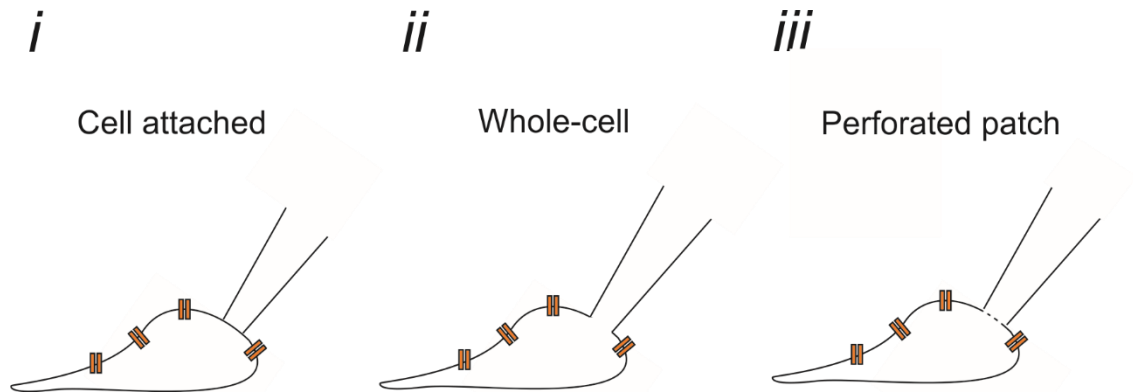


Figure 2.3. Patch-clamp configurations used in this project.

Enlargement of area in the red circle in Fig. 2.1. Patch-clamp configurations: *i* Cell-attached, *ii* Whole-cell, *iii* Perforated patch. The V_m is clamped through the injection of current. The reference electrode allows changes in the current passing through the membrane (mediated by ion channel opening) to be detected as a change in potential difference between the reference and pipette electrode. Individual ion channels are represented by the pairs of orange squares.

Whole-cell patch-clamp recordings allow the activity of all open ion channels on the plasma membrane (the macroscopic current) to be investigated using a patch clamp set up (Fig. 2.2). Once in cell-attached mode, suction is applied to break open the plasma membrane and gain access to the intracellular milieu. The intracellular solution is clamped by the diffusion of the pipette solution into the cell. Thus, a period of a few min has to be given from breaking in to allow complete exchange of solution [384,385].

The whole-cell configuration allows large currents to be recorded. Whole-cell patch-clamp can be used in both the voltage-clamp and the current-clamp configurations to measure current and V_m , respectively. In voltage clamp, the V_m is set at a defined value and current is injected to maintain it. The amplitude of the injected current is determined by the current passing through the circuit (from patch electrode to bath

electrode through the cell membrane). This compensatory current varies depending on the permeability of the membrane (which is dependent on the active ion channels present) [382,386]. In this way the activity of the ion channels in the plasma membrane can be monitored; the more ion channel activity the more current needs to be injected to maintain a constant V_m .

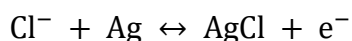
When measuring whole-cell currents it is important to compensate for the patch pipette resistance and the access resistance. Uncompensated resistance creates an offset in the voltage [382]. Additionally, the capacitance of the circuit can also be compensated for, thus removing the capacitive peaks. The capacitance observed is mainly due to the cell membrane as the borosilicate glass that the patch pipette is made of has a low dielectric constant (i.e. a low capacity to store electric charge in an electric field) [110].

The perforated patch configuration can be used to study whole-cell currents and the membrane potential of cells. Rather than breaching the plasma membrane using suction access is achieved through the perforation of the membrane by antibiotics (such as amphotericin B) with which the pipette solution is supplemented. These perforations allow the passage of monovalent cations across the membrane while preventing nucleotides and intracellular components to pass [380,381]. As the cell membrane is not breached the intracellular environment is maintained in a condition closer to physiological conditions than during the whole-cell [380,381,386]. As the presence of amphotericin B in the pipette solution impairs gigaohm seal formation [383,387], a barrier of pipette solution not containing amphotericin B is required. This was achieved by dipping the empty pipette in the pipette solution (not supplemented with amphotericin B), allowing some solution to enter the pipette through capillary action. The pipette was then back-filled with the amphotericin B

supplemented solution. Once a gigaseal was formed the set up was left for <15 min to allow amphotericin B to reach the membrane and perforate it. Effective electrical access to the cell, as indicated by the capacitative peaks, was observed by stepping the voltage from 0 mV to 10 mV for 10 ms once every 3 min.

2.7.1 Specific feature of the patch-clamp set up used

All patch-clamp current recordings were performed using an Axopatch 200A amplifier (Axon Instruments, USA) controlled by GePulse software (Dr Pusch, CNR, Italy, <http://users.ge.ibf.cnr.it/pusch/programs-mik.htm>) with an analogue-to-digital and digital-to-analogue converter (USB-6221, National Instruments, UK). Pipettes were pulled from borosilicate glass capillary tubes (Harvard Apparatus, USA) using a PC-10 pipette puller (Narishige, Japan). Pipette tip diameter yielded a resistance of 2-3 MΩ in the majority of the working solutions. AgCl electrodes were used for the reference and pipette electrodes. The AgCl electrodes consist of a silver wire (0.25 mm thick) that has an outer coat of AgCl. The ionic current from the cell is converted to an electrical current within the metal wire according to the following reaction:



which occurs at the interface between the liquid and the metal.

The pipette was positioned onto the cell membrane by using a micromanipulator (Scientifica, UK). Cells were visualised under a IX51 inverted microscope (Olympus, UK). Fluorescence signal was observed with a microscope fluorescence filter (GFP) within the microscope, while illuminating the cells with 470 nm light (generated by Dual OptoLED power supply (Cairn Research, UK)). All equipment was placed on

an anti-vibration table (Newport, USA) surrounded by a Faraday cage to provide stability to the equipment and electrical shielding, respectively [382]. The electrical noise was reduced through the grounding of all equipment through a ground distributor, while the measuring electrode was grounded to the headstage. Surge protectors were used to eliminate external “switch noise” which might be caused by drops and surges in the local power grid. In whole-cell recordings the series resistance was usually compensated to >70% to achieve a maximal effective series resistance lower than 5-10 M Ω . Experiments were conducted at 20-22°C. The cell capacitance was assessed by measuring the area under the capacitive transient elicited by a 10 mV voltage step or using the cell capacitance compensation circuit of the amplifier. To compare currents from cells of different sizes, current amplitude was normalised for cell capacitance. Currents were filtered at 2 kHz and sampled at 10 kHz. The signal is filtered using a low-pass anti-aliasing filter to “clean” it from extraneous noise.

2.7.2 Main electrophysiological protocols and analysis

The solutions used for the electrophysiological recordings are described in Section 2.13. All electrophysiological recordings were made at ~22 °C.

Current versus blue light intensity

Heterologous ChR2, ChR2(H134R) and iChloC currents were recorded with the whole-cell configuration of the patch-clamp technique in HEK-293T. ChR2(H134R) currents were recorded from aortic, mesenteric and pulmonary SMCs isolated from ChR2(H134R)-SM mice. The V_m was recorded from aortic SMCs isolated from control or ChR2(H134R)-SM mice using the perforated patch-clamp configuration during current-clamp. Cells were exposed to 2 s pulses of blue light (470 nm

wavelength) with intensities ranging from 0.01 mW/mm² to 12.1 mW/mm² unless stated otherwise. A Dual OptoLED power supply (Cairn Research, UK) was used for these stimulations.

Heterologous ChR2, ChR2(H134R) and iChloC currents were measured at -80 mV. The photocurrent *versus* blue light intensity relationship was fitted with the Hill equation of the form:

$$I = \frac{I_{max}}{1 + \left(\frac{EL_{50}}{light\ intensity} \right)^h}, \quad \text{Eq. [2.2]}$$

where I is the steady-state current density measured at a given light intensity, I_{max} is the maximal asymptotic current reached, EL_{50} is the light intensity causing half-maximal current activation and h is the slope coefficient. ChR2 and the ChR2 mutants were assumed to be closed in the dark. Based on this, the small current observed in the dark was subtracted offline from I at each light intensity. Thus, I represents the opsin mediated light-activated current.

V_m versus blue light intensity protocol and analysis

The V_m *versus* light intensity relationship was fitted with a modified Hill Equation of the form:

$$V_m = V_{rest} + \frac{V_{max} - V_{rest}}{1 + \left(\frac{EL_{50}}{light\ intensity} \right)^v}, \quad \text{Eq. [2.3]}$$

where V_m is the membrane potential, V_{rest} is the resting V_m (in the absence of light stimulation), V_{max} is the maximal asymptotic V_m reached, EL_{50} is the light intensity causing half-maximal membrane depolarisation and v is the slope coefficient.

LVGCC current protocol and analysis

The whole-cell current density *versus* voltage relationship for LVGCCs was obtained by applying 200 ms pulses from -70 mV to +60 mV in 5 mV steps from a holding potential (V_{hold}) of -60 mV. The peak current density was measured for each V_m and plotted *versus* V_m . The resulting relationship was fitted with a modified Boltzmann equation of the form:

$$I_p = G_{\text{max}} \times \frac{(V_m - E_{\text{rev}})}{\left(1 + \exp\left(\frac{V_m - V_{0.5,a}}{s_a}\right)\right)}, \quad \text{Eq. [2.4]}$$

where I_p is the peak current density, G_{max} is the whole-cell Ca^{2+} conductance, E_{rev} is the whole-cell current reversal potential, $V_{0.5,a}$ is the midpoint voltage for current activation, and s_a is the e-fold slope.

Steady-state inactivation properties were assessed by applying 1.5 s conditioning pre-pulses from -90 mV to 40 mV in 10 mV steps. Each pre-pulse was followed by a 200 ms test-pulse to 10 mV. The V_{hold} was -60 mV. The peak current (I_p) in response to each test-pulse was measured, normalised for the maximal I_p and plotted *versus* the V_m of the pre-pulse. The resulting steady-state inactivation was fitted with a Boltzmann equation of the form:

$$I_p = \frac{I_{\text{max}}}{\left(1 + \exp\left(\frac{V_m - V_{0.5,h}}{s_h}\right)\right)}, \quad \text{Eq. [2.5]}$$

where I_{max} is the maximal current, $V_{0.5,h}$ is the midpoint voltage for current inactivation and s_h is the e-fold slope.

Recovery protocol

A double-pulse protocol was used to determine the time required for the amplitude of ChR2 and ChR2(H134R) currents to recover following inactivation during a 2 s pulse of blue light of 0.08 or 12.1 mW/mm². The conditioning pulse was followed by a varying recovery period (3 s to 55 s) and a subsequent 2 s test pulse of blue light of 0.08 or 12.1 mW/mm². The extent of recovery was expressed as the ratio of the peak current measured during a test pulse relative to that measured during the conditioning pulse.

ChR2 ion selectivity

The selectivity of ChR2(H134R) and iChloC to Cl⁻ and H⁺ was assessed by determining the shift in E_{rev} of the currents when [H⁺]_e or [Cl⁻]_e were changed through alteration in the pH or by replacing the extracellular Cl⁻ with an equimolar concentration of gluconate.

2.7.3 Solution exchange

For some experiments, the extracellular solution needed to be rapidly exchanged. This was achieved using a perfusion system composed of 8 borosilicate tubes (0.8 mm inner diameter) connected reservoirs of the different solutions required. The patch-clamped cells were lifted off the surface of the tissue culture dish and placed shallowly into the perfusion tubes. During the recording of the current the cell was moved between the different perfusion tubes.

2.7.4 Junction potential measurements

Liquid junction potentials appear at the interface between solutions of different composition. One such interface is between the pipette solution and the bath

solution. They stem from the accumulation of charge due to the concentration gradients and the relative motility of the different ions [388]. If not compensated for, the liquid junction potentials produce an offset between the command voltage and the voltage the cell is clamped at. In whole cell experiments, the liquid junction potential was compensated before the pipette touched the cell (as described in [389,390]). In experiments where the bath solution was exchanged during the course of the current recording, the liquid junction potentials were calculated using the Patcher's Power Tools (Dr. Francisco Mendez, Department of Membrane Biophysics at MPI Biophysical Chemistry in Göttingen, Germany; <http://www3.mpibpc.mpg.de/groups/neher/index.php?page=software>) and corrected offline. In these experiments, an agar bridge was also used to avoid different ions coming into contact with the Ag/AgCl pellet in the reference electrode.

2.8 *Wire myography*

Contraction of aortic, mesenteric and pulmonary artery rings isolated from control or ChR2(H134R)-SM mice was measured using a small vessel dual wire myograph 410A system (Danish Myo Technology, Denmark). This isometric recording technique relies on the passing of two wires through the lumen of the vessel that are then connected to jaws that can be separated to stretch the artery ring. The tension is detected by a force transducer attached to one of the jaws [384,385], see Figure 2.4.

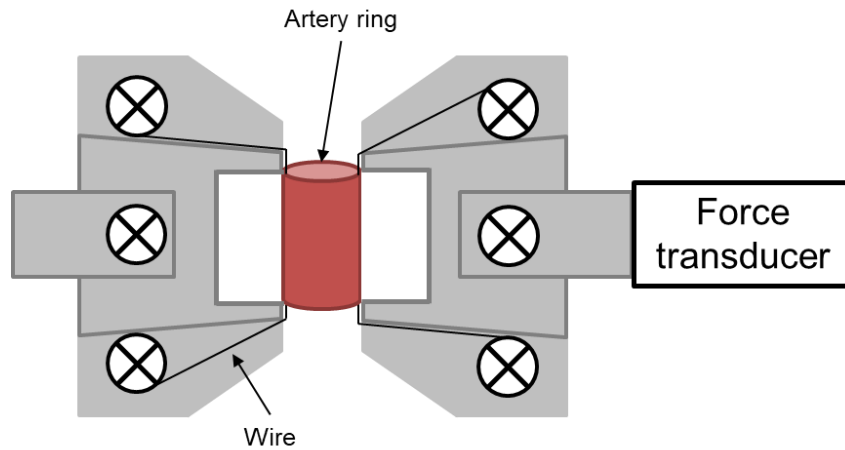


Figure 2.4. Wire myograph schematic.

An artery ring is shown mounted using wires passed through the lumen. The wires are secured to the myograph through the tightening of the screws. The tension of the artery ring is detected by the force transducer connected to one of the jaws.

2.8.1 Normalisation procedure

At the start of the isometric tension recording the artery ring must be under an adequate basal level of tension corresponding to a physiological transmural pressure (100 mmHg for the aorta, 70 mmHg for the mesenteric artery (MA) and 30 mmHg for the pulmonary artery (PA)) [391–393]. These values were termed IntCirc100, IntCirc70 and IntCirc30. The specific separation of the jaws required to reach these values was determined using a custom normalisation procedure script in IGOR (written by Dr Tammaro). The normalisation procedure script is underpinned by the Laplace law. The tension (force per unit length of the vessel wall, mN/mm) experienced by the wall is related to the transmural pressure (P_i) estimated to stretch the vessel to the IntCirc required for the artery type, and internal circumference (IC) of the artery by the Laplace equation:

$$Tension = P_i \times \frac{IC}{2\pi} \quad \text{Eq. [2.6]}$$

Artery rings (~2 mm in length) were isolated from the descending thoracic aorta, second order mesenteric artery and second order pulmonary artery from control mice or Chr2(H134R)-SM mice and cleaned from connective tissue using fine forceps and butterfly scissors. For some experiments the endothelial layer was mechanically removed from the artery rings. This was done through passing horse hair (taken from the mane) through the lumen of the artery ring. Artery rings were then mounted on a wire myograph using tungsten wires (diameter 25-40 μm (Goodfellow, UK)) and submerged in PSS solution at 37 °C [143,392,393]. Immediately after being mounted in the wire myograph, the length of the artery rings was measured using a microscope camera (BestScope, China) with an inbuilt graticule mounted on a Nikon SMZ645 microscope (Nikon Instruments, UK). The artery rings were then left for 30 min to equilibrate. After this the artery rings were stretched to produce a tension of 0.1 mN and left for 90 s before steady-state tension was recorded. The artery ring was then stretched in increments of either 100 μm or 50 μm for the aorta or the mesenteric and pulmonary artery rings, respectively, and the tension recorded after 90 s before proceeding with the next incremental increase in tension. This was done until the tension recorded was 8-10 mN for aortic rings or 4-5 mN for mesenteric and pulmonary rings. Tension was recorded using AD/DA converter (USB-6221, National Instruments, UK) and Chart v5 (Strathclyde University, UK) software. From the tension (mN), wire thickness, desired transmural pressure and the length of the artery ring the appropriate level of baseline tension was found using IgorPro software (Wavemetrics, USA). The full

stretch/tension normalisation was done for all artery segments prior to commencement of myography experiments.

2.8.2 Myography experimental procedures

Before the start of the experiment, the response of the artery rings to stimuli was tested through the raising of the extracellular concentration of KCl ($[KCl]_e$) by 50 mM for 6 min. This stimulation was repeated twice to achieve stable degrees of contraction. Following washout and recovery of baseline tension, the tissue was exposed to periods of blue light or pharmacological agents.

The tension *versus* blue light intensity relationships in the absence of PE were fitted with the Hill equation of the form:

$$T = \frac{T_{ss}}{1 + \left(\frac{EL_{50}}{\text{light intensity}} \right)^s}, \quad \text{Eq. [2.7]}$$

where T is the tension (normalised for length of the artery ring) measured at a given light intensity. T_{ss} is the maximal asymptotic tension (normalised for length) reached, EL_{50} is the light intensity causing half-maximal tension and s is the slope coefficient.

A Hill equation of this form was also used to quantify the tension *versus* $[KCl]_e$ relationship. In this case the independent variable was $[KCl]_e$.

As described in Chapter 5, the response of isolated aortic rings to PE was characterised by a rapid contraction followed by a transient relaxation and a slow increase in tension. Thus, PE did not induce in a stable level of contraction. To account for this changing level of tension, in the experiments conducted in the

presence of PE (Chapter 4, Figure 4.10), the effect of each light stimulation on vessel tension was expressed relative to the mean of the tension measured immediately before and after a given blue light exposure. A custom LED blue light source was used to stimulate the tissue with blue light of various intensities (0.01-7.21 mW/mm²).

The tension *versus* blue light intensity relationship in the presence of PE was fitted with a modified Hill equation of the form:

$$T_n = 1 + \frac{T_{ssn} - 1}{1 + \left(\frac{EL_{50}}{\text{light intensity}} \right)^{s_n}}, \quad \text{Eq. [2.8]}$$

T_n is the normalised tension at a given light intensity, T_{ssn} is the asymptotic tension recorded, EL_{50} is the light intensity causing half-maximal tension and s_n is the slope coefficient.

Myography data were recorded with the Chart software (Dr Dempster, Strathclyde University) and analysed with Igor Pro (Wavemetrics, USA).

2.9 **Confocal imaging**

Confocal microscopy was used to estimate the cellular distribution of ChR2(H134R) in freshly isolated vascular SMCs (aortic, mesenteric and pulmonary).

Freshly isolated live SMCs (aortic, mesenteric and pulmonary) were placed on a glass-bottomed 35 mm petri dish and allowed to settle for 10 min prior to imaging. Imaging was done at ~22 °C. Individual cells were imaged using a LSM510 META confocal laser scanning system (Zeiss, UK) connected to an inverted AxioVert 200

microscope with a 63X objective (Zeiss, UK) and controlled using the Zen 2012 (Blue edition) software (Zeiss, UK). A drop of Zeiss Immersol oil was added to the microscope objective prior to placing the chamber in the microscope. The refractive index of the Immersol oil is similar to that of the glass bottom of the chamber (~1.5 in both cases). The high refractive index aids the resolving power of the microscope and thus provides a more accurate recording of fluorescence signal. To reveal the cellular localisation of the ChR2(H134R)-eYFP in isolated SMCs, an excitation wavelength of 514 nm was used and an emission wavelength of 522 nm was recorded. All confocal images were acquired with identical settings. Cellular distribution of eYFP was estimated via fluorescence intensity plots of cross section taken from these images. The fluorescence intensity of each cross sections was normalised for the maximal intensity measured within the same cross section.

2.10 Epifluorescence imaging

Relative expression levels of ChR2(H134R)-eYFP in SMCs isolated from ChR2(H134R)-SM mice were assessed using epifluorescence microscopy at ~22 °C and quantified as outlined in [375,394]. The fluorescence of each aortic, mesenteric and pulmonary SMC was normalised for the cell surface area (fluorescence density) and averaged. Fluorescence density of mesenteric and pulmonary SMCs was expressed relative to the fluorescence density of aortic SMCs. All epifluorescence images were acquired using identical settings with an AxioCam MRm camera (Zeiss, UK) connected to an Axio Vert.A1 inverted microscope with a 10X objective (Zeiss, UK), using Zen 2012 (Blue edition) software (Zeiss, UK).

2.11 *pH imaging*

Freshly isolated aortic SMCs were AM-loaded with 1 mg/ml cSNARF1 (Thermo Fisher, USA) (10 min) before plated on a glass-bottomed chamber placed in the field of view of a Zeiss LSM confocal system. cSNARF1 is a lipid soluble, membrane permeable, fluorescent dye that loses its membrane permeability once hydrolysed by esterases inside the cell. Thus, any dye entering the cell will remain there. The cells were then perfused for 3 min with “External ChR” solution (see Section 2.13.1 for recipe) to remove excess fluorescent dye before imaged for pH ratiometrically (excitation 555 nm, emission 640 and 580 nm). Fluorescence was measured before and after a 7 min blue light (470 nm) stimulation of varying intensity (0.03 mW/mm² to 2.4 mW/mm²). The fluorescence ratio was converted to the intracellular pH (pH_i) with a calibration curve determined in separate experiments described below (Section 2.11.1). The innate buffering capacity of the aortic cells was also established as described below (Section 2.11.2). These experiments were conducted at ~22 °C.

2.11.1 *Nigericin calibration*

For analysis of the ratiometric recordings a calibration curve was necessary. This was constructed using the nigericin calibration technique [395,396]. Nigericin is a K⁺/H⁺ antiporter. Incubating SMCs with 20 μM nigericin and then raising the [K⁺]_e to 140 mM (~the same as the [K⁺]_i in SMCs) the pH_i becomes clamped to the pH_e. Thus, changing the extracellular pH (pH_e) leads to changes in pH_i. Superfusing the SMCs with different pH high K⁺ solutions will thus provide reference values for the ratiometric signal at different pH_i values. Calibration was done using aortic SMCs isolated from four ChR2(H134R)-SM mice. Recording the intracellular fluorescence

ratio (R), a relation between the pH_i (which is set by the pH_e already measured using a pH-meter) and fluorescence can be obtained and fitted with a sigmoidal function of the form:

$$pH = pKa + \log_{10} \left(\frac{R_{max} - R}{R - R_{min}} \right) + pF \quad \text{Eq. [2.9]}$$

Where R_{max} is the maximum fluorescence ratio corresponding to an overwhelming ratio of protonated to unprotonated cSNARF molecules (normally detected at pH 5); R_{min} is the minimum fluorescence ratio corresponding to an overwhelming ratio of unprotonated to protonated cSNARF molecules (normally detected at pH 9); pKa is the apparent dissociation constant for the protonated cSNARF; and pF is the negative logarithm of the ratio of fluorescence at pH 9 to pH 5 measured at 640 nm. The pH_e of the solutions used were between pH 6 and pH 8.5.

2.11.2 Buffering capacity measurements

The buffering capacity is the capacity of a cell to buffer changes in pH_i following addition or removal of H^+ and is defined as $\Delta[H^+]_i/\Delta pH_i$. The total buffering power is defined as the amount of acid or base needed to produce 1 pH unit change in 1 litre of cytosol [397]. The buffering capacity of the isolated aortic SMCs was ascertained through acid load experiments. In brief, the SMCs were loaded with cSNARF, as described above, before the chamber was perfused with “External ChR” solution modified to contain 110 mM NaCl and 30 mM NH_4Cl . After the pH_i stabilised (~3 min) the perfusate was changed to a “External ChR” solution, modified to contain 125 mM NaCl and 15 mM NH_4Cl . This was repeated twice more with solutions containing 135 or 140 mM NaCl and 5 or 0 mM NH_4Cl .

This NH_4Cl induced acid load of the SMC relies on differential membrane permeability and incomplete ionic dissociation of NH_4Cl (see Fig. 2.5). In the perfusate the NH_4Cl is dissociated into NH_4^+ and Cl^- . The weak base, NH_4^+ , in turn undergoes partial dissociation into NH_3 and H^+ . Subsequently rapid alkalinisation of the cytosol is observed due to the entry of uncharged NH_3 and its subsequent intracellular protonation. The charged NH_4^+ gradually enters the cells, possibly via K^+ channels and dissociates into NH_3 and H^+ reducing pH_i from the initial alkalinisation. This causes the intracellular NH_4^+ concentration to increased. Removing the NH_4Cl from the perfusate causes a rapid acidification of the cytosol. The intracellular NH_3 readily exits the cell, causing the intracellular NH_4^+ to dissociate into NH_3 and H^+ . The slow exit of the H^+ released at this point leads to acute acidification. The greater the NH_4^+ load achieved during the perfusion the greater this acidification is. The pH_i recovers through the activation of mechanisms such as the sodium proton exchanger (NHE).

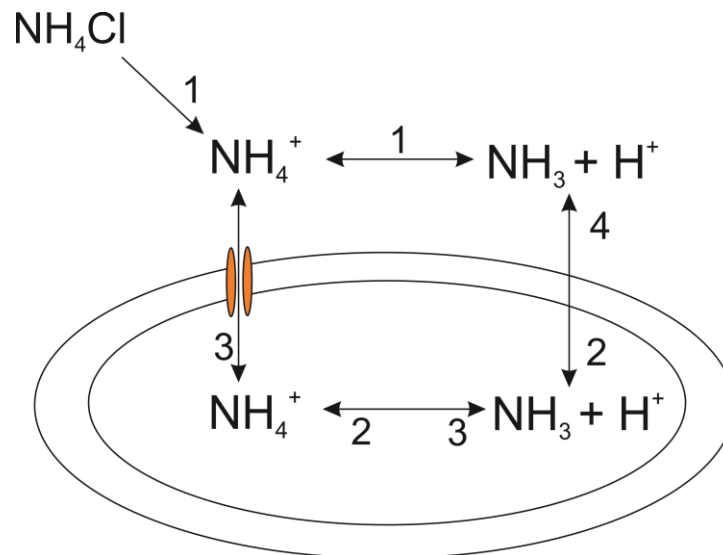


Figure 2.5. Diagram of the theory of acid load buffering capacity.

The buffering capacity of the isolated live aortic SMCs was ascertained through the addition and gradual removal of the weak base NH_4^+ to cause varying degrees of acid load. 1:

Addition of NH_4Cl leads to an increase in $[\text{NH}_4^+]$ which dissociates. 2: NH_3 enters the cell and associates with intracellular H^+ to form NH_4^+ . This leads to a reduction in pH_i . 3: NH_4^+ can slowly enter the cell via K^+ channels (as shown in orange in the figure) which shifts the equilibrium for NH_4^+ dissociation within the cell to be more favourable to produce NH_3 and H^+ . This can manifest itself as a gradual decrease in pH_i . 4: Once the extracellular NH_4Cl is removed or reduced the NH_3 diffuses out of the cell leading to a prominent reduction in pH_i .

2.12 External pH measurements

Extracellular pH (pH_e) was measured in close proximity to isolated artery rings from control and ChR2(H134R)-SM mice using microelectrodes, at $\sim 22^\circ\text{C}$. The measuring and reference microelectrodes were connected to the pH/mV-meter (Unisense, Denmark). The microelectrodes were calibrated using three pH meter calibration buffers (pH 4, pH 7 and pH 10) before use. Isolated aortic rings were placed in a modified physiological salt solution (see section 2.14.2) and the reference electrode was placed in the same solution. The pH microelectrode was then placed in close proximity (gently touching the artery wall). Following establishing of a stable baseline the pH_e was recorded for 2-5 min before application of blue light stimulus (2.4 mW/mm^2) for 7 min after which another 2-5 min were recorded.

2.13 Patch-clamp electrophysiology solutions

2.13.1 Opsin mediated photocurrents

In experiments aimed at recording whole-cell ChR2(H134R) current specifically, the intracellular and extracellular solutions had ionic components close to the expected physiological ionic concentrations. These solutions therefore allowed currents

carried by the majority of the physiological charge carriers to be observed. The solutions used are outlined below.

External ChR solution

Ion/compound	Concentration (mM)	Comments
KCl	5.4	
NaCl	140	
MgCl ₂	1	
CaCl ₂	1.8	
HEPES	10	Buffering agent
Glucose	10	Nutrient

pH 7.4 with NaOH

Internal ChR solution

Ion/compound	Concentration (mM)	Comments
K-Aspartate	80	Provides physiological [Cl] _i and [K ⁺] _i
KCl	50	
MgCl ₂	1	
MgATP	3	
EGTA	10	Ca ²⁺ chelator
HEPES	10	Buffering agent
CaCl ₂	3 (3.8x10 ⁻⁵)	
NaCl	10	

pH 7.4 with KOH, free [Ca²⁺]_i shown in parenthesis as calculated using MAXCHELATOR (<http://maxchelator.stanford.edu/index.html>).

For perforated-patch experiments, the Internal ChR solution was supplemented with 0.3 mg/ml amphotericin B (Merck, UK) and MgATP was omitted (the free [Ca²⁺]_i in

this solution was 35 nM as calculated using MAXCHELATOR). The amphotericin B was made fresh each day. Amphotericin B was dissolved in DMSO to make a 30 mg/ml stock. This was sonicated for 8 min before it was used for the preparation of the pipette solution. The pipette solution contained 0.3 µg/ml amphotericin B and was sonicated for 8 min before use. The prepared solution was kept in the dark and on ice when not used.

For the measurement of the hyperpolarising potential of iChloC mediated currents, the pipette solution was modified to more accurately reflect the lower $[Cl^-]_i$ observed in cell types other than SMCs.

Internal iChloC solution

Ion/compound	Concentration (mM)	Comments
K-Aspartate	130	Provides physiological $[Cl^-]_i$ Ca ²⁺ chelator Buffering agent
MgCl ₂	1	
EGTA	10	
HEPES	10	
CaCl ₂	3 (3.6x10 ⁻⁵)	
NaCl	5	

pH 7.4 with KOH, free $[Ca^{2+}]_i$ shown in parenthesis as calculated using MAXCHELATOR (<http://maxchelator.stanford.edu/index.html>).

2.13.2 Proton current reversal potential measurements

For the study of the proton permeability of ChR2(H134R) and iChloC, the reversal potential of the blue light induced current was measured. In these solutions the main physiological permeant ions (apart from H⁺) were replaced or excluded. The $[H^+]_e$

was altered through changing the pH_e to pH 5, pH 7.4 or pH 9. Furthermore, the solutions were supplemented with 10 μ M DIDS to block endogenous acid activated ion channels [398,399].

External H^+ solution

Ion/compound	Concentration (mM)	Comments
NMDG	147.8	Impermeant positive ion
HCl	30	
Gluconic acid	118	Replaces Cl^-
BaCl ₂	2	K^+ channel blocker
MgCl ₂	1.3	
Glucose	11	Nutrient
HEPES	20	Buffering agent

pH 5, pH 7 and pH 9 adjusted with Gluconic acid or TEA-OH.

Similarly, the intracellular solution was optimised to reduce currents carried by ions other than H^+ .

Internal H^+ solution

Ion/compound	Concentration (mM)	Comments
NMDG	128	Impermeant positive ion
HCl	10	
Gluconic acid	118	Replaces Cl^-
EGTA	10	Ca^{2+} chelator
HEPES	20	Buffering agent

pH 7.2 with CsOH. The free $[Ca^{2+}]_i$ was anticipated to be nominal.

2.13.3 Anion current reversal potential measurements

For the study of the anion current through ChR2(H134R) and iChloC, extracellular solutions in which the main anion was altered were used. This was achieved by

replacing the NaCl in External ChR solution (see Section 2.13.1) with an equimolar quantity of Na-gluconate. The intracellular solution used in these experiments was the Internal ChR solution (also described in Section 2.13.1)

2.13.4 LVGCC recordings

These solutions were used to eliminate the K^+ currents; Ba^{2+} , Cs^{2+} and tetraethylammonium (TEA) block K^+ channels [400–403]. $BaCl_2$ was included to increase the current observed as Ba^{2+} has greater conductance through LVGCCs than Ca^{2+} [404].

External LVGCC solution

Ion/compound	Concentration (mM)	Comment
NaCl	104	
CsCl	5.4	K^+ channel blocker
TEA-Cl	20	K^+ channel blocker
$BaCl_2$	5	K^+ channel blocker and charge carrier
$MgCl_2$	1	
NaH_2PO_4	1	
Glucose	10	Nutrient
HEPES	5	Buffering agent

pH 7.4 with NaOH.

Internal LVGCC solution

Ion/compound	Concentration (mM)	Comment
CsCl	112	K^+ channel blocker
Na_2ATP	3	
HEPES	5	Buffering agent
EGTA	10	Ca^{2+} chelator
$MgSO_4 \cdot 7H_2O$	1	

pH 7.4 with NaOH. The free $[\text{Ca}^{2+}]_i$ was anticipated to be nominal.

2.14 pH solutions

2.14.1 Intracellular pH solutions

Measurement of pH_i was done in the presence of External ChR solution as described in Section 2.13.1. However, the buffering capacity of the isolated aortic SMCs and the calibration curve required other solutions. They are as follows:

pH_i calibration buffer

Ion/compound	mM	Comment
KCl	140	
MgCl ₂	1	
EGTA	0.5	Ca ²⁺ chelator
HEPES	5	Buffering agent
MES	5	Buffering agent

pH ~6.0, 6.5, 7.0 or 7.5 with KOH

Buffering potential solutions

Ion/compound	mM	Comment
KCl	5.4	
MgCl ₂	1	
CaCl ₂	1.8	
HEPES	10	Buffering agent
Glucose	10	Nutrient
NaCl	140-X	

pH 7.4 with NaOH

Varying amounts (represented by X in the above table) of NaCl was replaced with equimolar NH₄Cl to establish the buffering capacity of the isolated aortic SMCs. The NH₄Cl concentrations used were 0 mM, 5 mM, 15 mM and 30 mM.

2.14.2 Extracellular pH

Extracellular pH (pH_e) was measured in isolated artery rings bathed in the solutions below. Of note is the reduced amount of buffering compound (HEPES).

pH_e solution

Ion/compound	mM	Comment
NaCl	100	Buffering agent
MgCl ₂	5	
CaCl ₂	1.8	
HEPES	1	

pH 7.4 with NaOH

2.15 Myography

Artery rings were isolated in PSS and myography experiments were conducted in PSS.

Physiological salt solution

Ion/compound	mM	Comment
NaCl	122	Buffering agent
KCl	5	
HEPES	10	
KH ₂ PO ₄	0.5	
NaH ₂ PO ₄	0.5	
MgCl ₂	1	
Glucose	11	Nutrient

CaCl ₂		1.8	
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pH 7.4 with NaOH

In some experiments, the PSS was modified to either exclude the CaCl₂ or replace NaCl (with equimolar NMDG-Cl).

2.16 **Data analysis**

Electrophysiological data was analysed using ANA (Dr M. Pusch, CNR, Italy) or IGOR (Wavemetrics, USA). Myography data was analysed using IGOR. ImageJ (National Institutes of Health, USA) software was used for analysis of imaging data. The two-tailed Student's T-test or ANOVA with Bonferroni's post-hoc test was used for statistical analysis as appropriate. Data are presented as mean±S.E.M alongside the number of experiments (n). Each set of experiments involving HEK-293T cells was composed of >4 different complete experiments (a complete experiment being represented by an independent cell passage, transfection followed by an independent patch clamp recording day). All experiments were carried out in cells passaged between 5 and 30 times. All experiments involving native cells or isolated artery rings, involved samples obtained from >4 mice. The number of experiments reported in the text correspond to the total number of cells or tissue samples used for each set of experiments. Differences were considered significant if $p < 0.05$.

2.17 *Experimental design*

The work described in this thesis adheres to the guidelines summarised in “Experimental design and analysis and their reporting: new guidance for publication in BJP.” [379].

2.17.1 Justification of animal species and strain used

This project involved the use of mice for cell and tissue studies. The use of human samples was not possible due to low availability and inability to genetically modify the tissue. The use of freshly isolated SMCs from mouse arteries was necessary as cultured cells may have remodelled expression of the various ion channels. Computer simulations of SMCs expressing ChR2 could not be used for this study as no such models exist. However, it could be anticipated that the work presented in this thesis will provide essential information for the generation of these models. This, in the future, will help reducing and, to an extent, replacing the use of animals in research.

Mice were used because they represent a well-established model for cardiovascular physiology, thus enabling comparison of my results with those already published in the literature. Mice share physiological and biochemical properties with humans; thus, the observations made using this model provides insight into human physiology and biochemistry.

2.17.2 Randomisation and blinding

It was not feasible for the operator not to know the genotype of the mice used during each individual experiments as the absence or presence of the effects assessed (such as the ionic current or force of contraction), immediately revealed to the

operator the genotype of the sample under examination. Methods of analysis were established during study design, and prior to execution of the experiments, to remove possible operator bias. For each genotype, the animals were selected randomly on the day of the experiment.

2.18 *Contribution to work*

The data presented in this thesis were acquired by myself, Nils Jonas Gunne Rorsman (NJGR), with contributions from other members of the lab as outlined in this section. Experiments were designed by NJGR and Dr Paolo Tammaro (PT). Custom scripts for analysis of the data were written by NJGR and PT. Preliminary work on the vascular optogenetic mouse model was conducted by Dr Hannah Garnett (HG) and Dr Chau My Ta (CMT) (both former PhD students in the Tammaro Group). CMT also assisted in collecting the data for Figure 4.3 (~50%) and Figure 4.8 (25%).

Chapter 3

Characterisation of Channelrhodopsin-2 for use in vascular optogenetics

3.1 Introduction

The aim of this chapter was to systematically compare the electrophysiological properties of ChR2 and the ChR2 variant ChR2(H134R) expressed in HEK-293T cells. These channels are commonly used as depolarising tools in the study of neurones and more recently in the study of cardiac cells [267,405]. To determine their suitability to be used in a mouse model for vascular optogenetics, ChR2 and ChR2(H134R) were characterised in a heterologous expression system. It was found that both ChR2 and ChR2(H134R) mediated blue light activated inward depolarising currents that were stable in response to continuous light stimulation for several minutes. The magnitude of the current density, however, was significantly larger in cells transfected with ChR2(H134R) compared to ChR2, suggesting that ChR2(H134R) may provide more significant depolarisation during vascular optogenetics. The pharmacological properties of ChR2 or ChR2(H134R) have not yet been investigated. To utilise these channels in vascular optogenetics in conjunction with modulators of vessel tone, it is important to examine the effect that these modulators may have on ChR2(H134R) directly. Thus, in this chapter the sensitivity of cloned ChR2(H134R) to TMEM16A and LVGCC blockers was studied, as these blockers are utilised in the work described in the subsequent studies (Chapter 5). It was found that ChR2(H134R) is insensitive to these agents. These observations indicate ChR2(H134R) as a suitable opsin for the generation of transgenic mice for use in vascular optogenetics.

3.2 Results

3.2.1 Blue light stimulation results in depolarising currents

The study began by examining the whole cell current amplitude in response to a broad range of blue light intensities. The steady-state current increased as the blue light intensity was increased. Individual HEK-293T cells may have different cell surface area and a different number of expressed channels. To compensate for this variation, whole-cell current was normalised for the cell capacitance (which is proportional to the cell surface [382,406]) to obtain the current density. The whole-cell current was measured in HEK-293T cells transfected with ChR2 or ChR2(H134R) at -80 mV. Cells were exposed to blue light for 2 s separated by 4 s periods in the dark to allow a return to basal current. As previously reported, currents in ChR2 and ChR2(H134R) transfected HEK-293T cells were characterised by a rapid activation followed by a rapid inactivation to a new steady-state level (Fig. 3.1). The steady-state current increased as light intensity was increased. The fit of the steady-state current density *versus* blue light intensity relationships with Eq. 2.3 yielded a maximal asymptotic current (I_{max}) of ~ 9 pA/pF and ~ 22 pA/pF for ChR2 and ChR2(H134R), respectively (Fig. 3.1B). The light intensity causing half-maximal effect (EL_{50}) was ~ 1.1 and 0.2 mW/mm² for ChR2 and ChR2(H134R) respectively. The slope factor (h) was ~ 0.6 for ChR2 and 0.8 for ChR2(H134R) (Table 3.1).

Increasing the intensity from 0.01 mW/mm² to 12.1 mW/mm² resulted in an increase in current from 0.6 ± 0.1 pA/pF ($n=7$) to 7.1 ± 0.7 pA/pF ($n=7$) for ChR2 and 1.7 ± 0.3 pA/pF ($n=16$) to 21.1 ± 1.9 pA/pF ($n=16$) for ChR2(H134R). In contrast, there was essentially no current increase observed in non-transfected HEK-293T cells (Fig. 3.1). At a light intensity of 12.1 mW/mm², the measured current was limited to 0.2 ± 0.2 pA/pF ($n=5$) in non-transfected HEK-293T cells; these values were

approximately 90-fold smaller than the currents observed in ChR2(H134R) transfected cells and approximately 35-fold smaller than ChR2 transfected cells.

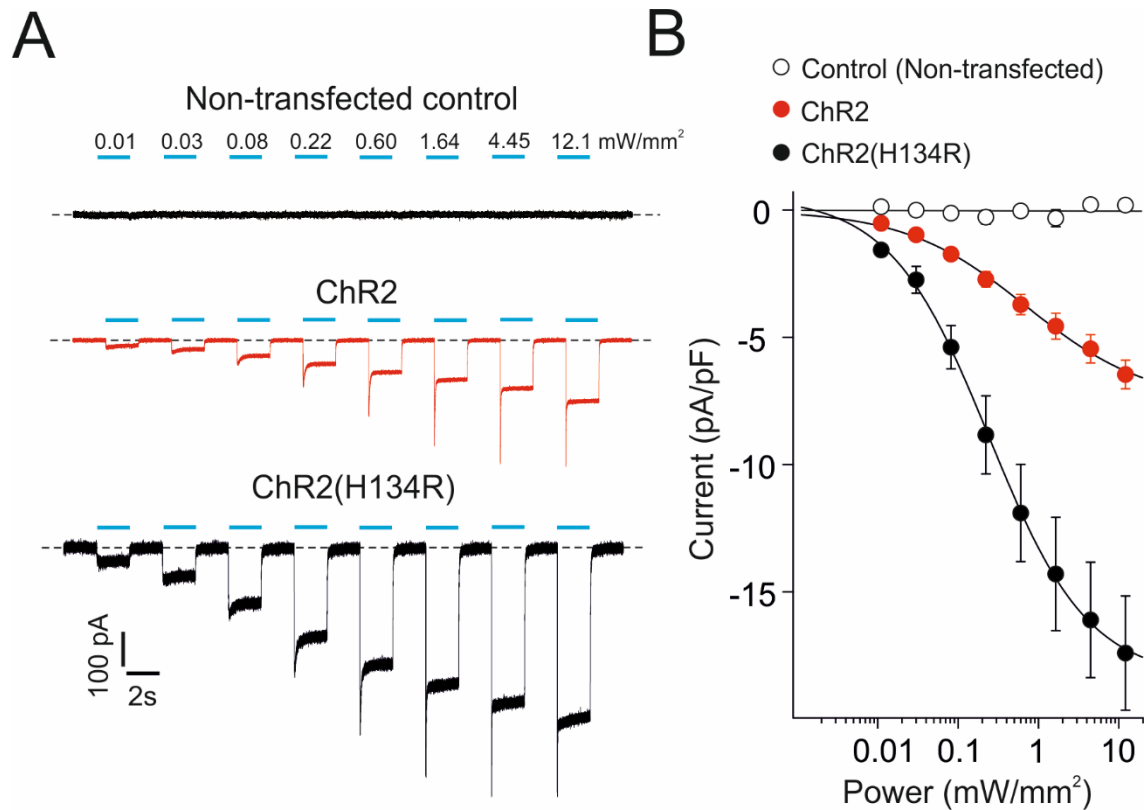


Figure 3.1. Electrophysiological analysis of ChR2 and ChR2(H134R) currents in the HEK-293T expression system.

A. Representative whole-cell currents recorded from non-transfected and ChR2 or ChR2(H134R) expressing HEK-293T cells, as indicated, in response to blue light stimulation (as indicated by the horizontal blue bars). The holding potential was -80 mV. The dashed horizontal lines represent the zero-current level. **B.** Mean whole-cell current density versus voltage relationships measured at the end of each light exposure for non-transfected and ChR2 or ChR2(H134R) expressing HEK-293T cells, as indicated. Smooth line through the red and black circles represent the best fit of the data with Hill equation (Eq. 2.3) with an EL_{50} of 1.1 ± 0.3 (ChR2) and 0.2 ± 0.1 (ChR2(H134R)). The number of experiments was 5, 7 and 16 for non-transfected control, ChR2 and ChR2(H134R), respectively. The line drawn through the white circles was drawn by eye.

Table 3.1. Parameters for the current versus blue light intensity relationships for HEK-293T cells expressing ChR2 or ChR2(H134R)-SM.

Parameter	ChR2	ChR2(H134R)
EL_{50}	1.1 ± 0.1 (7)	0.2 ± 0.1 (16)
h	0.6 ± 0.1 (7)*	0.8 ± 0.1 (16)*
max	-8.8 ± 0.9 (7)*	-22 ± 2.0 (16)*

Parameters (EL_{50} , h and I_{max}) extrapolated from the Hill fit (Eq. 2.3) of the current versus light intensity relationship for ChR2 and ChR2(H134R) expressed in HEK-293T cells. The number of cells is indicated in parentheses (n). Significant differences indicated by * ($p < 0.05$).

3.2.2 Time dependent recovery of a transient peak current in ChR2 and ChR2(H134R) expressing cells

Figure 3.1 indicated that the response of ChR2 and ChR2(H134R) to light has a complex time course. In response to a pulse of blue light, the current amplitude reached a peak and then decreased to a stable steady level. This transient peak current may represent fast channel inactivation. This issue was tested using a protocol previously exploited to study the inactivation properties of voltage-gated channels. Experiments to study the recovery from inactivation were conducted using both low and high blue light intensity stimulation (0.08 mW/mm^2 and 12.1 mW/mm^2 , respectively). A pre-pulse was followed by a test-pulse after an interval of varying duration (0.4 s to 55 s). In Figure 3.2B, the normalised peak transient current of the test pulse (normalised to the pre-pulse peak) is plotted *versus* the duration of the interval for 0.08 mW/mm^2 and 12.1 mW/mm^2 . Fitting the transient peak (from the start of the light activation to the beginning of the steady-state current) of the photocurrent with a single exponential function provided time constants of $9.2 \pm 1.5 \text{ s}$ ($n=5$) and $9.4 \pm 2.1 \text{ s}$ ($n=5$) for ChR2 and ChR2(H134R),

respectively, at 0.08 mW/mm^2 . The time constants at 12.1 mW/mm^2 were $8.2 \pm 0.9 \text{ s}$ ($n=5$) and $7.7 \pm 1.0 \text{ s}$ ($n=5$) for ChR2 and ChR2(H134R), respectively.

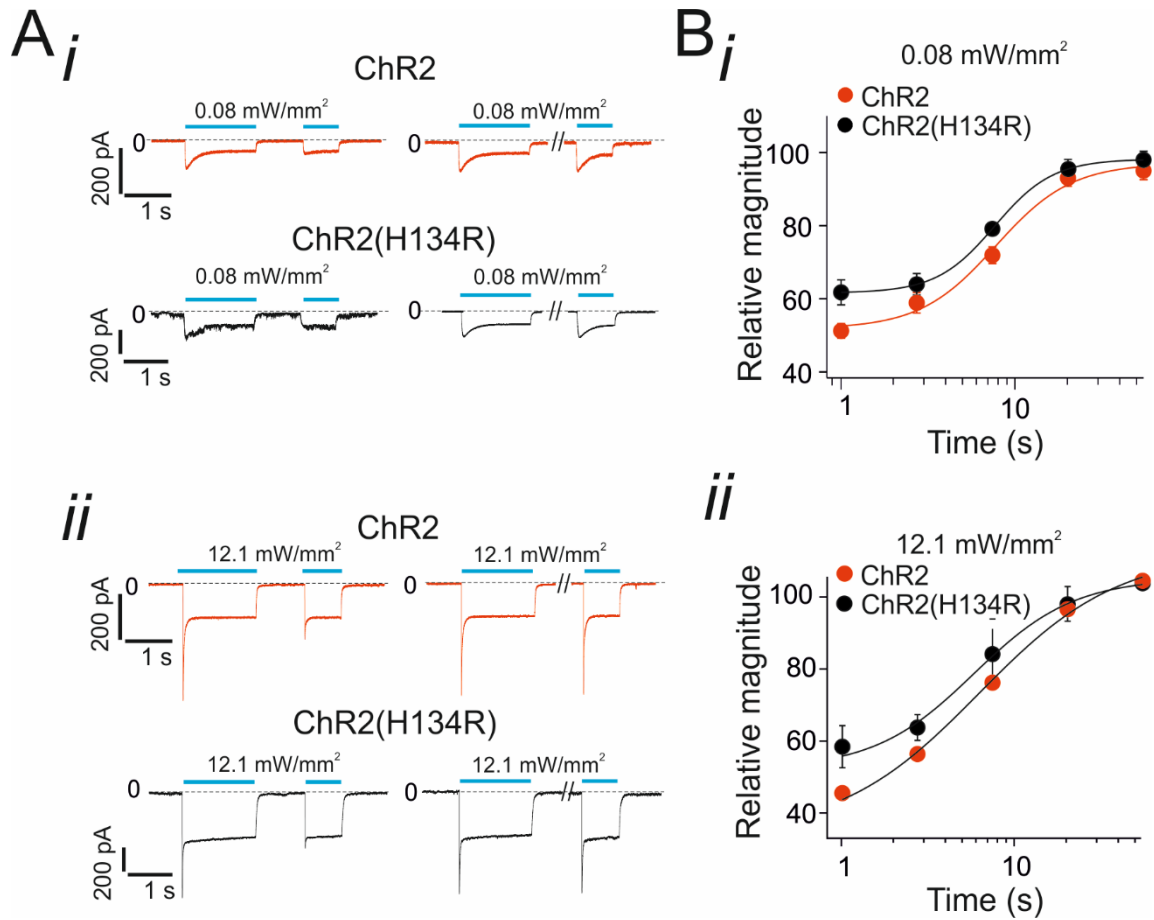


Figure 3.2. Recovery of transient peak current in ChR2 and ChR2(H134R).

A. Representative whole-cell currents elicited by blue light stimulation (as indicated by the horizontal blue bars) of varying intensity (0.08 mW/mm^2 (i) or 12.1 mW/mm^2 (ii)) in ChR2 or ChR2(H134R) expressing HEK-293T cells. The holding potential was -80 mV . Zero current level (dark current) is indicated by the dashed lines. The longer recovery period (54 s) (represented by //) is omitted for clarity. **B.** The relative magnitude (%) of the test-pulse peak current compared to the pre-pulse peak current is shown against the recovery time for both 0.08 mW/mm^2 (i) and 12.1 mW/mm^2 (ii). The number of experiments was 5 in all cases.

3.2.3 Extended blue light exposure leads to maintained photocurrents mediated by ChR2 and ChR2(H134R)

Vascular SMCs contract slowly. For example, contraction of isolated arteries in response to a depolarising agent (such as elevated extracellular KCl) require 3-10 min to fully develop [143,362]. Thus, the application of optogenetics to control vascular tone requires an opsin that can be active for several minutes. ChR2(H134R) and ChR2 have been most commonly used for experiments where the stimulation periods are in the milliseconds or seconds, as these are sufficient to trigger excitation of excitable cells such as neurons [262,407].

Thus, an essential requirement to the development of vascular optogenetics was to define the ability of ChR2 and ChR2(H134R) to maintain stable current in response to blue light stimulation maintained for several minutes. Therefore, ChR2 and ChR2(H134R) expressing HEK-293T cells were exposed to light of intensity 0.08 mW/mm² for 7 min at constant V_m (−80 mV) and the current recorded during whole-cell patch-clamp. This duration was chosen because, as shown in Chapter 4, 7 minutes is the time required for an isolated aortic ring to reach a stable contraction in response to elevated (50 mM) KCl.

Figure 3.3 shows that the photocurrent amplitude does not significantly change over extended blue light exposure periods. Mean ChR2 and ChR2(H134R) currents and the difference in amplitude are illustrated in Figure 3.3B. The steady state photocurrent elicited by 0.08 mW/mm² in HEK-293T cells expressing ChR2(H134R) or ChR2 saw small fluctuations (~0.8 pA/pF and ~0.2 pA/pF, respectively) in amplitude over the course of 7 minutes.

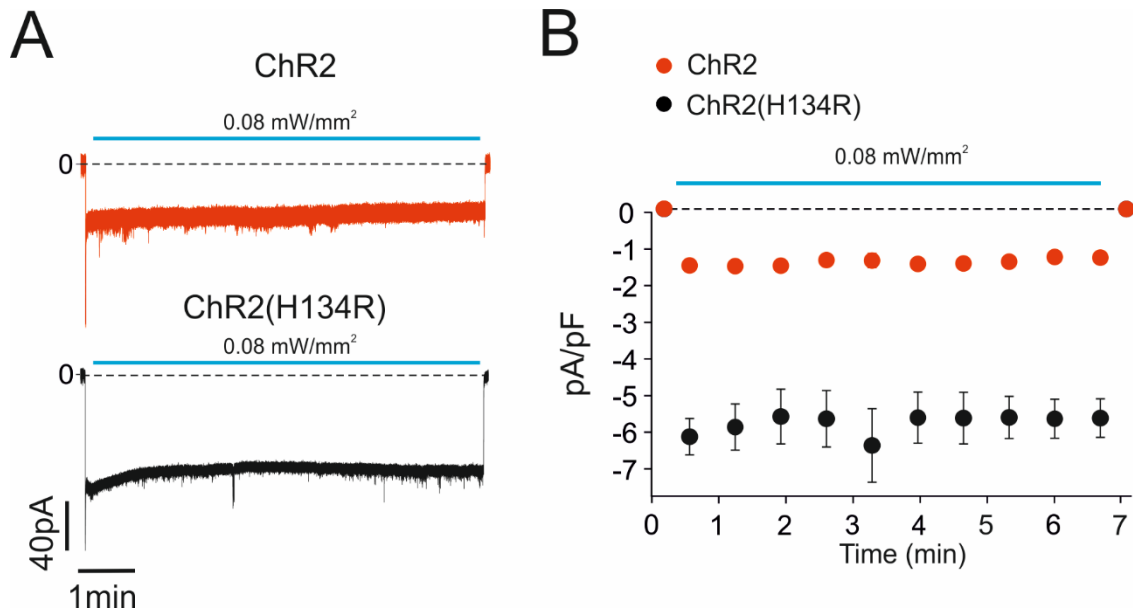


Figure 3.3. Effect of extended blue light exposure on current amplitude.

A. Representative whole-cell currents in HEK-293T cells expressing ChR2 (top) or ChR2(H134R) (bottom) evoked by extended exposure to blue light (represented by the horizontal blue bar). The holding potential was -80 mV. **B.** Mean current density of ChR2 and ChR2(H134R) throughout the extended exposure to blue light. The error bars for ChR2 are within the symbols. The number of experiments was 4 for ChR2 and 5 for ChR2(H134R).

3.2.4 Light dependent depolarisation of ChR2(H134R) expressing HEK-293T cells

To assess the capacity of ChR2(H134R)-mediated currents to produce membrane depolarisation, current-clamp patch-clamp recordings of the V_m in HEK-293T cells expressing ChR2(H134R) were performed. For ChR2(H134R) to be used in vascular optogenetics it must provide a sufficient depolarisation to cause LVGCC activation. Application of blue light of increasing intensity (from 0.01 mW/mm² to 12.1 mW/mm²) led to increasing levels of depolarisation (Fig. 3.4). A mean maximal depolarisation of 27 ± 2.0 mV ($n=12$) was observed in response to the maximal light intensity tested (12.1 mW/mm²), shifting the V_m from -35 ± 2.0 mV (measured in the dark) to -7.8 ± 1.3 mV ($n=12$). Cessation of light stimulation resulted in rapid return to resting V_m (Fig. 3.4A). The fit of the V_m versus blue light intensity relationship with Eq. 2.4 yielded an EL_{50} of 0.2 ± 0.1 mW/mm² ($n=12$), a slope factor (ν) of 0.7 ± 0.1 ($n=12$) and the maximal asymptotic V_m (V_{max}) of -7.0 ± 1.4 mV ($n=12$). In comparison,

no significant depolarisation was seen in the non-transfected control cells (the maximal deviation from resting V_m was 2.0 ± 0.4 mV at 12.1 mW/mm²) (n=5), not shown).

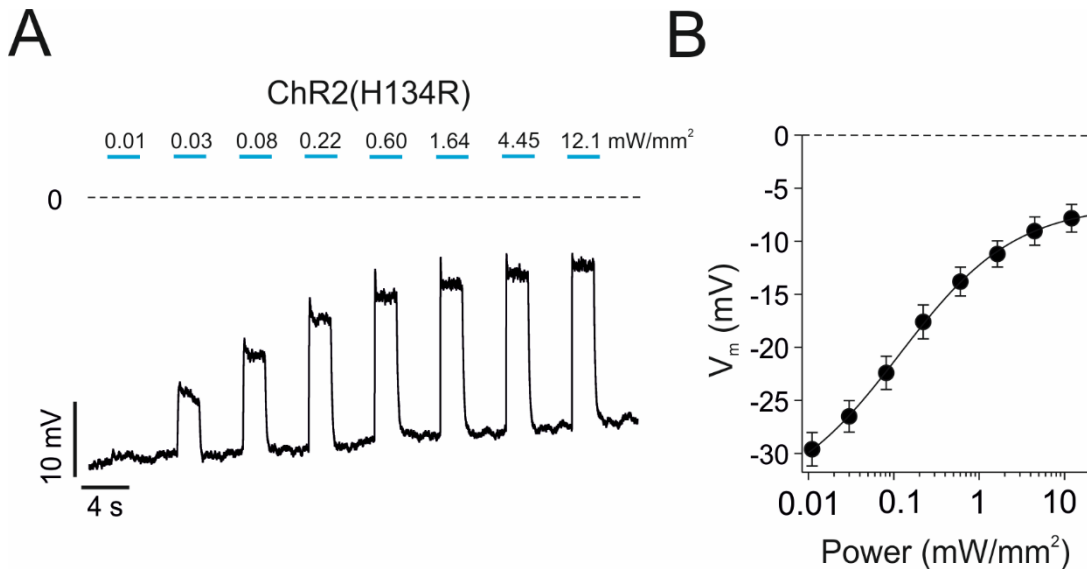


Figure 3.4. Blue light dependent membrane depolarisation.

A. Representative trace of light induced V_m depolarisation of a ChR2(H134R) expressing HEK-293T cell. The horizontal blue bars indicate the blue light application periods. The intensity of the blue light stimulations is noted above these blue bars. **B.** Mean depolarisation of ChR2(H134R) expressing HEK-293T cells (n=12). The curve through filled circles represents the best fit of the data with Eq. 2.4.

3.2.5 ChR2(H134R) pharmacology

To use ChR2(H134R) in a mouse model to establish and characterise vascular optogenetics, it was necessary to understand whether pharmacological compounds commonly used to study vascular function would alter the ChR2(H134R) mediated currents. To investigate whether this was the case, whole-cell patch-clamp recordings of blue light induced currents in ChR2(H134R) transfected HEK-293T cells were conducted while perfusing with pharmacological agents. ChR2(H134R) expressing HEK-293T cells displayed inward currents upon application of blue light (0.60 mW/mm²) while clamped at -80 mV. During this experiment, the cell was

perfused with “External ChR” solution (see Chapter 2, Section 2.11 for composition) on its own or supplemented with the non-selective Cl^- channel blocker DIDS (100 μM) or the CaCC selective blockers MONNA (3 μM) or Ani9 (1 μM). These compounds were applied for 10-20 s. The effect of the blocker on the blue light induced current was quantified as the ratio between the current measured in the absence of the drug and the current measured in the presence of the drug. As seen in Figure 3.5, none of the Cl^- channel blockers caused a change in the ChR2(H134R) mediated current.

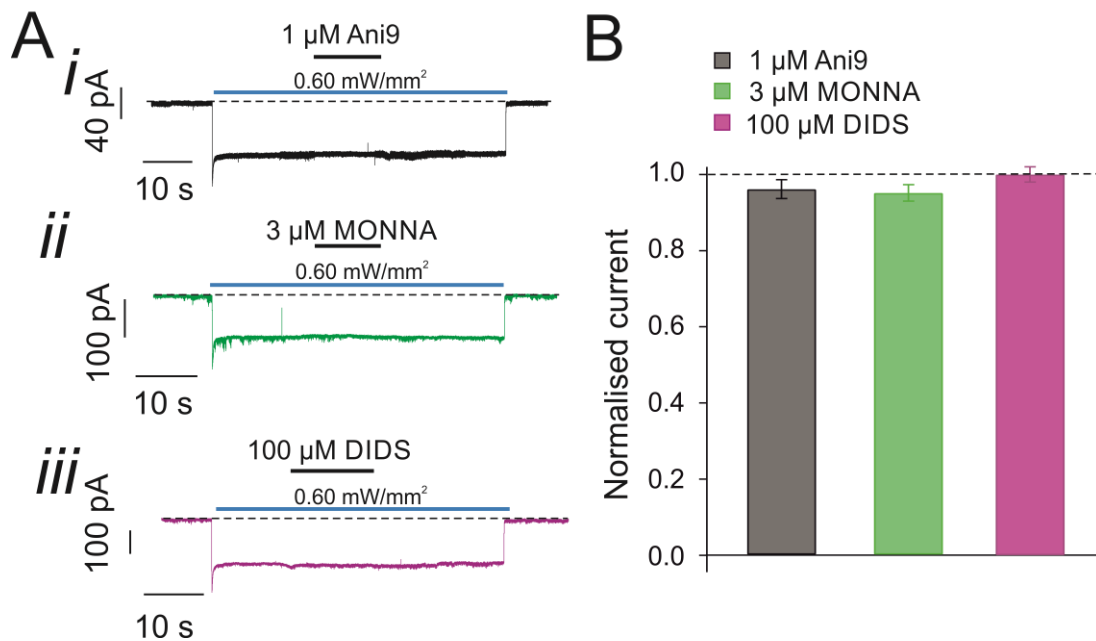


Figure 3.5. Effect of Cl^- channel blockers on ChR2(H134R) mediated current.

A. Representative whole cell recordings of HEK-293T cells expressing ChR2(H134R). Blue light stimulation (0.60 mW/mm²) is indicated by the horizontal blue bars whereas the application of the pharmacological agents is represented by the black horizontal bars (Ani9, *i*; MONNA, *ii*; DIDS, *iii*). The holding potential was -80 mV. **B.** Mean current during exposure to blue light and pharmacological agents, normalised to the current seen during exposure to blue light in the absence of the compounds (before and after). The number of experiments was 5-6 in each case.

In addition, experiments where the sensitivity of ChR2(H134R) mediated currents to nifedipine were also conducted. Nifedipine acts primarily on the LVGCCs at concentrations of ≤ 1 μM . Whole-cell patch-clamp experiments (conducted as

described above for the CaCC blockers) showed that a 10-20 s application of nifedipine did not affect the photocurrent amplitude exhibited by ChR2(H134R) expressing HEK-293T cells (in response to 0.60 mW/mm^2 at a V_{hold} of -80 mV) (Fig. 3.6). The pharmacology of opsins is poorly defined and the work presented in this thesis is the first time the direct effect of a variety of agents on ChR2(H134R) has been tested.

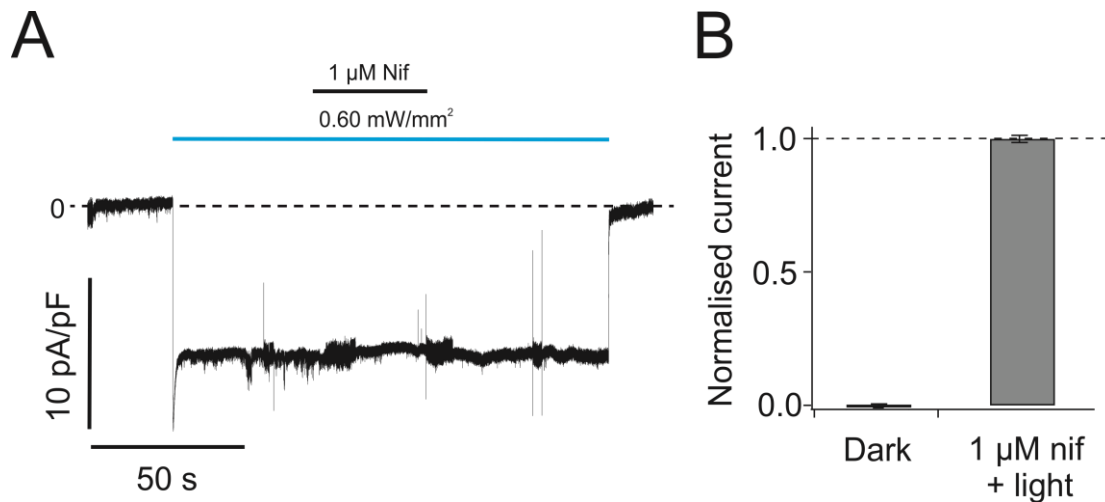


Figure 3.6. Effect of nifedipine on ChR2(H134R) mediated current.

A. Representative recording of current density of a HEK-293T cell expressing ChR2(H134R). Blue light stimulation (0.60 mW/mm^2) is indicated by the horizontal blue bar and the application of nifedipine is represented by the black horizontal bar. The holding potential was -80 mV . **B.** Mean current during the dark and during the exposure to blue light and nifedipine ($1 \mu\text{M}$), normalised to the current seen during exposure to blue light (dashed horizontal line). The number of experiments was 6.

3.3 Discussion

The main findings of this chapter were:

- 1) heterologously expressed ChR2 and ChR2(H134R) channels were activated by blue light over a range of light intensities but ChR2(H134R) provided a significantly greater amplitude photocurrent than ChR2;

- 2) heterologously expressed ChR2 and ChR2(H134R) required similar recovery time to regain the transient peak current;
- 3) ChR2 and ChR2(H134R) is stably activated by long exposure to light indicating suitability for vascular optogenetics;
- 4) ChR2(H134R) caused reliable depolarisation of HEK-293T cells in response to blue light stimulation;
- 5) ChR2(H134R) was not affected by commonly used pharmacological agents.

3.3.1 Light causes depolarising currents in ChR2 and ChR2(H134R) expressing cells that remain stable over time

For opsins to be used in a mouse model for vascular optogenetics, the relationship between the intensity of the blue light and the amplitude of the current elicited must be precisely defined. Knowing the sensitivity to blue light will enable fine control over the depolarisation induced by the photocurrent and thus, presumably, fine control of the artery tone. Both ChR2 and ChR2(H134R) presented stable inward currents that increased in amplitude with higher light intensities. The stability of the photocurrent is a highly important characteristic as vascular tone is controlled on the minute to hour rather than on a millisecond to second time scale [143,362]. The effect of ChR2(H134R) mediated currents led to a ~25 mV depolarisation of the cells. This degree of depolarisation in vascular SMCs would shift the V_m into a range where LVGCCs would be active and thus allow light induced contraction to occur. This made ChR2(H134R) a good candidate for use in vascular optogenetics. The capacity of the ChR2(H134R) mediated current to cause V_m depolarisation in SMCs is influenced by the input resistance of the cell. Vascular SMCs have high input resistance (~10 G Ω) e.g. [408]; this anticipates that even small currents are likely to

have a significant effect on the resting V_m . Thus it was likely that the ChR2(H134R) current would be sufficient to cause significant depolarisation of isolated SMCs. Small depolarisations (of less than 5 mV) already have an impact on voltage-dependent Ca^{2+} entry and smooth muscle contraction [126].

The depolarising potential of ChR2 was not measured as it was reasoned that it would likely be smaller than that mediated by ChR2(H134R) due to the lower amplitude photocurrents.

3.3.2 Time dependent recovery of fast phase current

The biphasic nature of the ChR2 and ChR2(H134R) mediated current was lost when repeatedly stimulating the cells. The recovery of the biphasic response was found to have similar time constants (τ) for both ChR2(H134R) and ChR2, with a full recovery was seen after ~20 s for both channels. The effect of this transient peak can be observed also in the V_m recordings as an initial peak depolarisation that quickly makes way for a steady state level. The current peak is unlikely to have an effect on the tone of most arteries because this transient current is very brief (~50-100 ms) relative to the longer time (~7 min) required for full contraction of vascular smooth muscle to occur. However, it cannot be excluded that this brief transient current may potentially result in action potential firing in the populations of SMCs which are readily excitable (e.g. in the portal vein [409], uterus and bladder (reviewed in [410])). This should be considered for future studies involving the use of opsins in these populations of SMCs. However, as most vascular SMCs are not excitable it can reasonably be assumed that the effect of the peak will be limited.

3.3.3 Pharmacological interactions with ChR2(H134R)

In the study of vascular physiology and pathophysiology, the mechanisms behind altered contraction is often studied using pharmacological antagonists or agonists of native ion channels or other signalling proteins. For vascular optogenetics to be a useful tool in these studies it is important to investigate the potential for these commonly used agents to act directly on the ChR2(H134R); any alteration in ChR2(H134R) mediated current and depolarisation due to drug interactions could be mistaken for an effect on the native channels. The experiments shown in Figure 3.5 and Figure 3.6 show that none of the four ion channel blockers tested affected the ChR2(H134R) mediated current. The compounds tested have been shown to act on their intended targets within one or two seconds (unpublished, Tammaro Group). Thus, the 10 s application periods were considered adequate. Although, DIDS, a generic chloride channel blocker, has been reported to require covalent bond formation to some Cl⁻ channel types [411–413], this effect is unlikely to occur on CaCCs because the inhibition of DIDS on CaCCs currents is fully reversible. Generally, many generic Cl⁻ channel blockers are not selective and act on cation channels as well [411]. Thus, novel, more specific blockers such as MONNA (which blocks both TMEM16A and TMEM16B) and Ani9 (which only blocks TMEM16A) have been used in this study. The potential interaction of these agents with ChR2(H134R) was studied as it could aid the dissection of the relative contribution of TMEM16A and TMEM16B to blue light induced contraction as studied in Chapter 5. The concentrations of the compounds used are those that cause full inhibition of cloned TMEM16A channels: MONNA and Ani9 at 3 μ M and 1 μ M, respectively [239,240]. The concentration of nifedipine used (1 μ M) was selected as it leads to complete blockage of LVGCCs, whilst limiting possible off-target effects on channels such as Kv2.1 [414] and Kv1.5 channels [415], which are also found in

SMCs. The pharmacological analysis of ChR2 could be expanded to include more ion channel agonists and antagonists as a part of a future project. Of particular interest would be to test the effect of modulators of TRP channels and the IP₃R (clotrimazole and 2-APB) used in Chapter 5 on ChR2(H134R). If these modulators were to block ChR2(H134R), the effect of TRP channels or IP₃R signalling on blue light induced contraction would be overestimated.

3.3.4 Conclusion

In this chapter, it was demonstrated that ChR2(H134R) mediates stable current in response to continuous blue light stimulus. ChR2(H134R) did not show any differences in the time dependent recovery of the transient peak. The transient peak in current resulted in a transient strong depolarisation before relaxing towards a steady-state V_m . While this is not an ideal scenario it is unlikely to be a major limitation as the transient peak (in both current amplitude and V_m) is completed within ~100 ms. Additionally, the pharmacological tested appeared to have no effect on the ChR2(H134R) mediated current. These results indicated that ChR2(H134R) was suitable for use in a mouse model of vascular optogenetics. This genetic modification will have multiple applications such as controlling blood flow and for drug discovery studies involving isolated SMC in which the effect on compounds can be studied at depolarised V_m .

Chapter 4

Generating a model for vascular optogenetics

4.1 **Introduction**

As outlined in greater detail in the introduction, optogenetics is an experimental approach involving the use of microbial opsins to modulate cell, tissue or organ function [258–260].

ChR2, as well mutant ChR2 variants with altered electrophysiological properties, have been used extensively as experimental tools in the field of neuroscience to define brain circuitry e.g. [262,268,269]. These opsins have also been harnessed to control the function of non-neuronal cell types. For example, ChR2 has enabled control of myocardial excitability and inotropy (e.g. [260,266,267]) and skeletal muscle contraction e.g. [270–272]. Optogenetics also has the potential to similarly transform vascular research. Thus, a mouse model expressing ChR2(H134R) selectively in SMCs was generated to allow the control of artery tone. The work described in this chapter was done to investigate the effect of ChR2(H134R) activation on currents, V_m and the tone of a variety of artery types.

During the course of my DPhil, some progress has been made in the field of vascular optogenetics by Wu *et al.* (2015), who selectively expressed a ChR2 mutant in murine SMCs to achieve optogenetic control of SMC. In their study, isolated aortic SMCs expressing ChR2 demonstrated light-induced currents in response to a single, high-intensity, blue light intensity. The tone of isolated mesenteric artery rings increased in response to high intensity blue light stimuli (ranging from 10 mW/mm² to 24 mW/mm²), which evokes maximal ChR2 activation [306,325]. Furthermore, it was possible to optogenetically modulate the perfusion pressure of the coronary and renal circulations in isolated hearts and kidneys [363]. More recently, optogenetics has been used to gain insights into the role of arterioles

and pericyte-covered capillaries in the control of cerebral blood flow [364]. These important studies have demonstrated the utility and potential of optogenetics in vascular research. Critically important mechanistic aspects, however, need to be addressed to enable a complete interpretation of vascular optogenetics experiments and to exploit this technology in the *in vivo* setting. To provide a more complete and comprehensive mechanistic appraisal of vascular optogenetics, it is critical to probe arterial responses over an adequately wide range of light stimulations to determine whether different degrees of ChR2 activation enable fine, graded control of vessel tone. Furthermore, it is well established that isolated wild-type mammalian systemic arteries pre-constricted with agonists, such as NA or PE, relax in response to blue light in a ChR2-independent manner [358,359,361]. Blue light-mediated relaxation has also been observed in murine cerebral arterioles *in situ* [360]. Thus, evaluating the capacity of ChR2-induced contraction to overcome the intrinsic blue light dependent arterial relaxation is an essential prerequisite for establishing vascular optogenetics *in vivo*, where the circulating levels of catecholamines (such as adrenaline and NA) will vary, depending on the extent of the sympathetic drive. This will include understanding the extent to which ChR2 opening provokes depolarisation and activation of endogenous channels as opposed to providing a direct source for non-physiological Ca^{2+} entry.

To address the issues outlined above, mice expressing ChR2(H134R) selectively in SMCs were generated. The effects of a wide range (0.01-12.1 mW/mm²) of blue light intensities on both the magnitude of ChR2(H134R) current in isolated vascular SMCs and the extent of contraction of artery rings were systematically examined. A broad range of artery type was included in these studies, namely: large conduit, small systemic and pulmonary arteries. In the case of isolated aortic SMCs and

isolated rings, experiments were extended to accurately map the effect of blue light on V_m and on the ionic mechanisms involved in contraction. It was found that ChR2(H134R) expression enabled optical voltage-clamp of these SMCs leading to LVGCC activation and artery contraction.

4.2 **Results**

4.2.1 *ChR2(H134R) expression and current density in vascular SMCs*

The project began by testing the degree of ChR2(H134R) expression in various artery types obtained from ChR2(H134R)-SM mice and control mice. Mice from these two genotypes did not differ in weight (at ~12 week of age, control and ChR2(H134R)-SM mice weighed 23.3 ± 0.7 g (N=20) and 23.9 ± 0.7 g (N=20), respectively) and no difference in mortality was observed. Assessing this point was important as the level of expression could influence the arterial sensitivity to blue light stimuli. Vascular SMCs (aortic, mesenteric and pulmonary) were isolated from control and ChR2(H134R)-SM mice, and imaged using confocal microscopy to determine ChR2(H134R) expression and distribution within the cell (Fig. 4.1A-C). SMCs obtained from ChR2(H134R)-SM mice had prominent eYFP signal demonstrating expression of the channel construct (Fig. 4.1A). The fluorescence signal was primarily detected in the plasma membrane of SMCs (4.1B), as indicated by the eYFP intensity profiles (solid white lines in Fig. 4.1A). In contrast, no detectable fluorescence was identified in vascular SMCs isolated from control mice (Fig. 4.1A).

I next aimed to obtain a measure of the total ChR2(H134R) expression in the various SMCs types. The average eYFP fluorescence intensity was assessed by

epifluorescence microscopy and normalised for cell area (fluorescence density). Fluorescence density varied between the different vascular SMCs types isolated from ChR2(H134R)-SM mice (Fig. 4.1C). The fluorescence density in mesenteric and pulmonary artery SMCs, relative to that of aortic SMCs, was 0.6 ± 0.1 ($n=49$) and 1.2 ± 0.1 ($n=64$), respectively (Fig. 4.1C). To explore the possibility that this was due to differences in cell surface area, the different SMC types were patch-clamped in the whole-cell configuration to determine their capacitance which is a measure of the cell surface area [110]. It was found that the various SMC types had comparable cell capacitance of 10.3 ± 1.0 pF ($n=14$, aorta), 13.4 ± 1.2 pF ($n=17$, mesenteric artery) and 10.4 ± 0.9 pF ($n=13$, pulmonary artery). Thus, it can be concluded that unequal levels of ChR2(H134R) expression occurred in the various SMCs types.

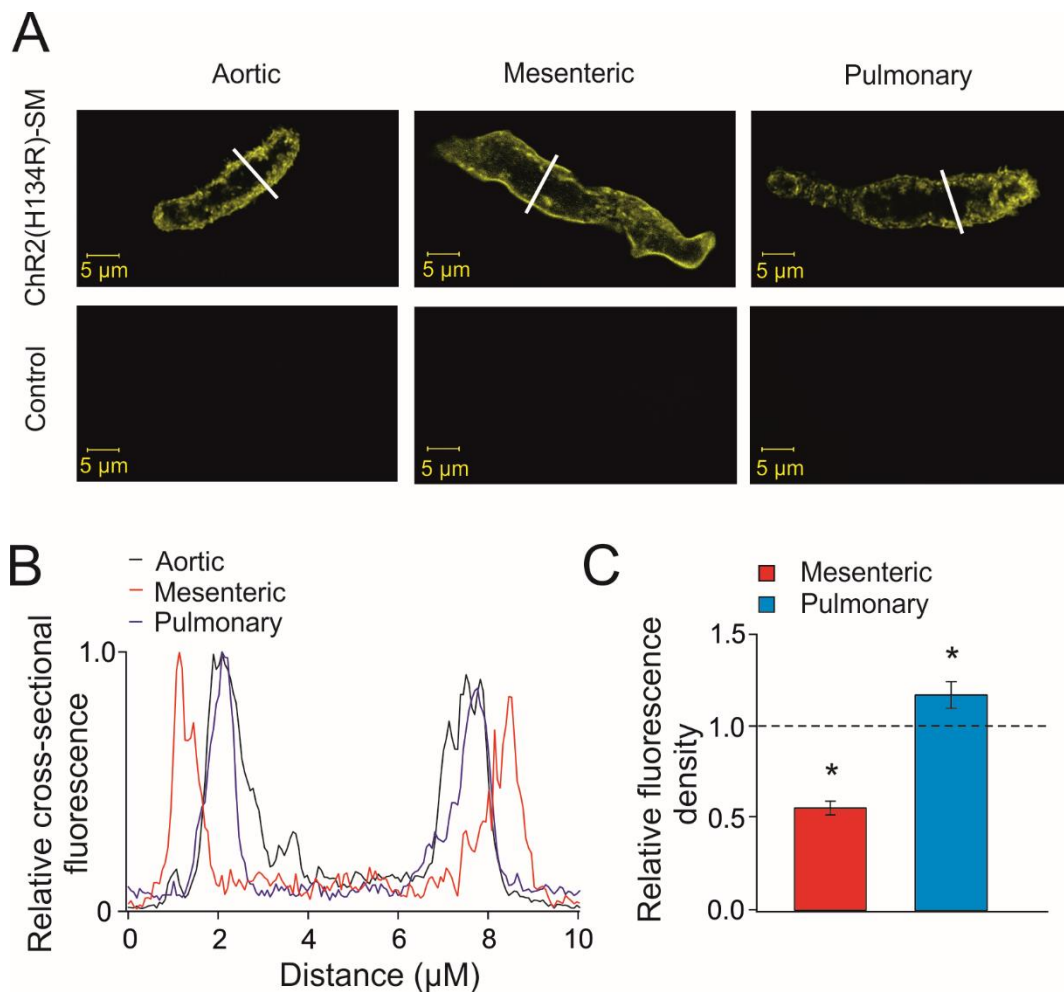


Figure 4.1. ChR2(H134R) expression in isolated vascular SMCs.

A. Representative confocal images of freshly isolated aortic, mesenteric and pulmonary SMCs obtained from control and ChR2(H134R)-SM mice, as indicated. Brightness was adjusted in these images to allow visual inspection of cell shape and expression distribution in all SMC types, otherwise all images were acquired with identical settings. **B.** Normalised fluorescence intensity of cell cross-section of aortic, mesenteric and pulmonary SMCs (cross-section location indicated by white bars in A). **C.** Average fluorescence density of mesenteric and pulmonary artery SMCs isolated from ChR2(H134R)-SM mice and expressed relative to the fluorescence measured in aortic SMCs ($n=49-64$).

To test if differences in ChR2(H134R) expression affected the current density, whole-cell currents were recorded from aortic, mesenteric and pulmonary SMCs isolated from ChR2(H134R)-SM mice. Similarly to ChR2(H134R) currents in heterologous expression systems [295,297,306], ChR2(H134R) currents in isolated vascular SMCs were characterised by a rapid activation followed by a rapid inactivation to a new steady-state level (Fig. 4.2A). The steady-state current increased as light intensity was increased. The fit of the steady-state current density *versus* blue light intensity relationships with Eq. 2.2 yielded a maximal asymptotic current (I_{max}) of ~ -18 pA/pF, ~ -13 pA/pF and ~ -24 pA/pF for aortic, mesenteric and pulmonary SMCs, respectively (Table 4.1). The light intensity causing half-maximal effect (EL_{50}) was ~ 0.3 mW/mm², and the slope factor (h) was $\sim 0.6-0.8$ in each case (Table 1). Thus, expression of ChR2(H134R) resulted in currents with different amplitudes (I_{max}), but of similar sensitivity to blue light, quantified in terms of the parameters EL_{50} and h . In contrast, there was essentially no current increase observed in SMCs obtained from control mice. For instance, the current in the presence of 12.1 mW/mm² blue light was 0.2 ± 0.1 pA/pF ($n=5$, aortic), 0.2 ± 0.1 pA/pF ($n=9$, mesenteric) and 0.3 ± 0.1 pA/pF ($n=6$, pulmonary) in SMCs obtained from

control mice; these values were approximately two orders of magnitude smaller than the currents observed in SMCs isolated from ChR2(H134R)-SM mice.

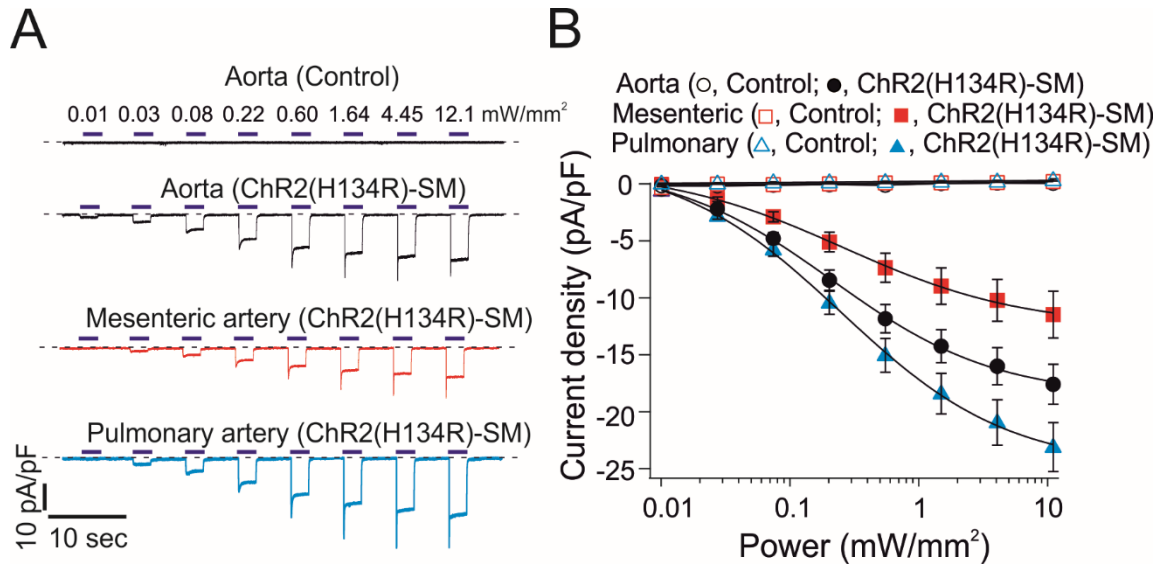


Figure 4.2. ChR2(H134R) current density in isolated vascular SMCs.

A. Whole-cell currents recorded from aortic, mesenteric and pulmonary SMCs isolated from control and ChR2(H134R)-SM mice, as indicated. Currents were recorded at -80 mV. SMCs isolated from control and ChR2(H134R)-SM mice were exposed to periods (2 s) of blue light of various intensities, as indicated by the horizontal blue bars. The dashed lines represent the level of current in the absence of light stimulation. **B.** Mean current density versus blue light intensity relationships for SMCs obtained from control ($n=6-9$) or ChR2(H134R)-SM mice ($n=12-14$), as indicated. The curves through the open symbols (control) were drawn by eye. The curves through the filled symbols represent the best fit of the data with Eq. 2.2. *, $P<0.05$ (One-Way ANOVA).

Table 4.1. Parameters for the current versus blue light intensity relationships for SMCs obtained from ChR2(H134R)-SM mice.

Parameter	Aorta	Mesenteric artery	Pulmonary artery
EL_{50} (mW/mm ²)	0.31±0.03 (n=14)	0.30±0.03 (n=12)	0.32±0.02 (n=13)
h	0.8±0.1 (n=14)	0.6±0.1 (n=12)	0.8±0.1 (n=13)

$I_{\max}$ (pA/pF)	-18 ± 2 (n=14)	-13 ± 2 (n=12)	-24 ± 2 (n=13)
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Parameters (EL_{50} , h and $I_{\max}$) extrapolated from the Hill fit (Eq. 2.2) of the current versus light intensity relationship for aortic, pulmonary and mesenteric vascular SMCs obtained from ChR2(H134R)-SM mice.

4.2.2 Effect of ChR2(H134R) activation on the V_m of isolated vascular SMCs

As outlined in the previous section, changes in arterial tone are expected to be linked to changes in V_m [126]. To quantitatively define the effects of ChR2(H134R) activation on the V_m of aortic SMC, the perforated-patch configuration in current-clamp mode was used. The resting V_m of ChR2(H134R)-SM aortic SMCs was -40.7 ± 1.9 mV (n=17), which was not significantly different from the resting V_m of vascular SMCs obtained from control mice (-45.0 ± 1.8 mV, n=9) (Fig. 4.3B). In response to light stimulations up to 12.1 mW/mm², the V_m depolarised to -19.2 ± 2.0 mV (n=17) in aortic SMCs isolated from ChR2(H134R)-SM mice (Fig. 4.3). Cessation of light stimulation resulted in return to resting V_m (Fig. 4.3A). The fit of the V_m versus light intensity relationship with Eq. 2.4 yielded an EL_{50} of 0.2 ± 0.1 mW/mm² (n=17), a slope factor (ν) of 0.6 ± 0.1 (n=17) and the maximal asymptotic V_m ($V_{\max}$) of -16.6 ± 2.2 mV (n=17). In contrast, the V_m of aortic SMCs obtained from control mice was not significantly affected by blue light exposure (Fig. 4.3A). Thus, the V_m of isolated aortic SMCs expressing ChR2(H134R) can be optically clamped by exposure to blue light.

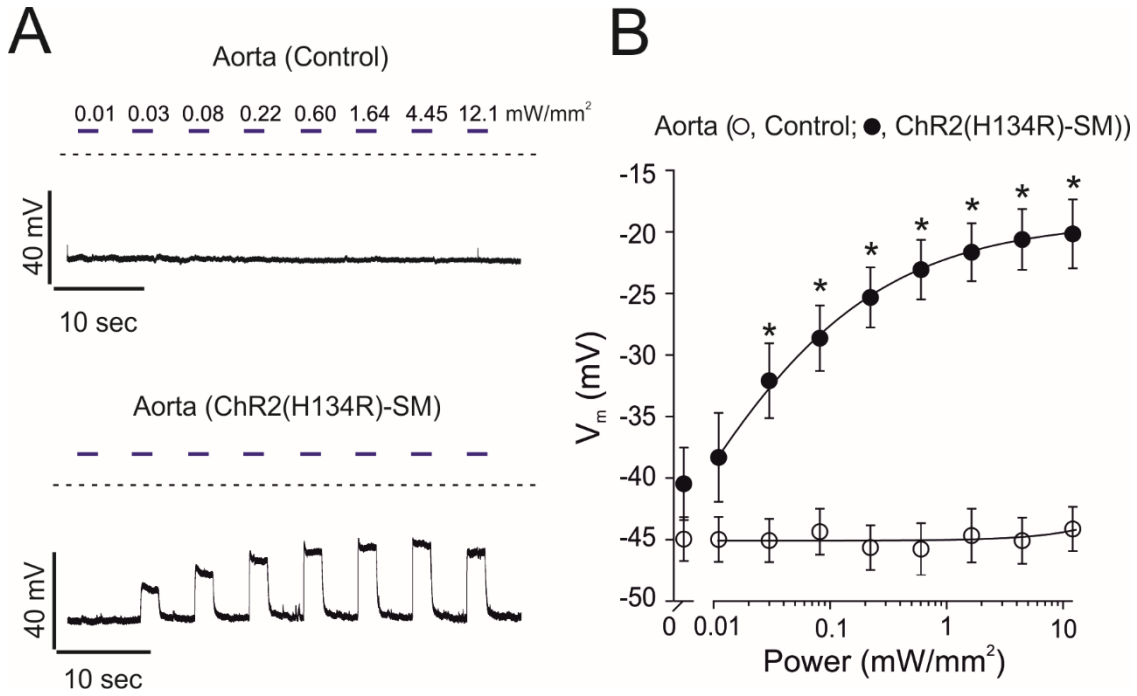


Figure 4.3. Effects of blue light on V_m in isolated aortic SMCs.

A. V_m recordings of aortic SMCs isolated from control and ChR2(H134R)-SM mice in response to blue light stimulations (2 s) of various intensities, as indicated by the horizontal blue bars. The dashed lines indicate the zero voltage level. **B.** Mean V_m versus blue light intensity relationships for aortic SMCs isolated from control ($n=9$) or ChR2(H134R)-SM mice ($n=17$). The curve through open circles (control) was drawn by eye. The curve through filled circles (ChR2(H134R)-SM) represents the best fit of the data with Eq. 2.3.

Under normal physiological conditions, V_m depolarisation of SMCs leads to activation of LVGGCs, increased Ca^{2+} influx and contraction e.g. [165,226,416]. To define the precise relationship between ChR2(H134R)-induced depolarisation and extent of LVGCC activation in our model, the voltage-dependence of LVGCC activation and inactivation were determined in aortic SMCs isolated from ChR2(H134R)-SM mice (Fig. 4.4). The steady-state activation curve was characterised by a half-activation potential ($V_{0.5,act}$) of -0.3 ± 1.0 mV ($n=13$) and a slope factor (s_a) of 8.5 ± 1.0 mV ($n=13$). The steady-state inactivation curve was characterised by a half-inactivation potential ($V_{0.5,inact}$) of -36.4 ± 0.8 mV ($n=8$) and a

slope factor (s_i) of 11.0 ± 0.7 mV ($n=8$). Window currents, the range of V_m in which LVGCCs are active, spanned between ~ -40 mV and ~ 10 mV (Fig. 4.4B). Thus, V_m depolarisation due to ChR2(H134R) activation shifted the V_m from a resting value of ~ -40 mV, to values that fall within the window current range of LVGCCs.

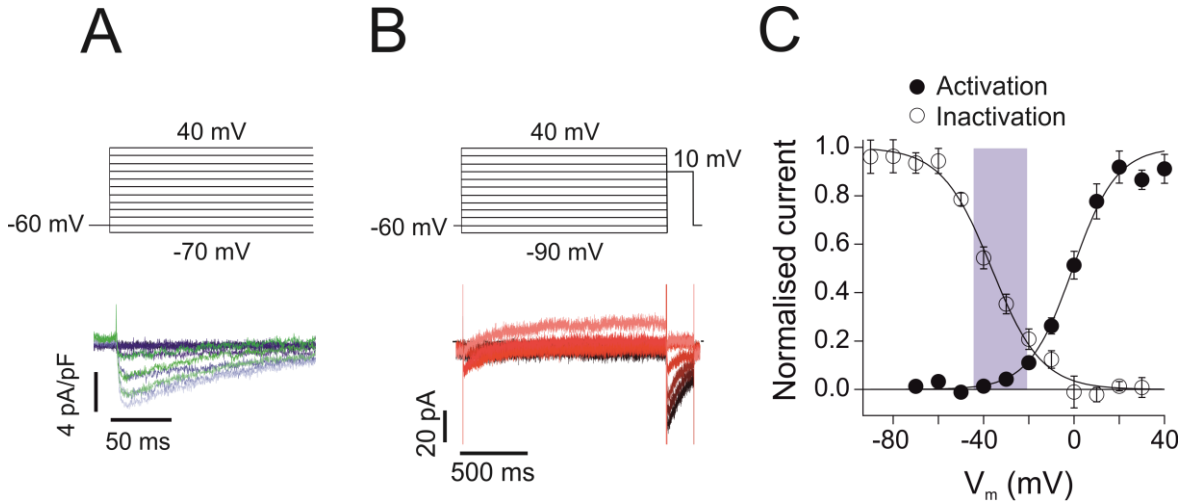


Figure 4.4. LVGCC currents in isolated aortic SMCs

A. Representative whole-cell LVGCC currents obtained from aortic SMCs isolated from ChR2(H134R)-SM mice in response to the activation stimulation protocol shown in the top panel. For clarity, only the currents elicited every 20 mV are displayed. The currents elicited by the different potentials have been coloured with progressively lighter shades of purple (starting with dark purple at -90 mV) going to progressively darker shades of green (ending in dark green at 30 mV). **B.** Representative whole-cell LVGCC currents obtained from aortic SMCs isolated from ChR2(H134R)-SM mice in response to the inactivation stimulation protocol shown in the top panel. For clarity, only the currents elicited every 20 mV are displayed. The currents elicited at different potentials have been coloured for clarity (going from black at -90 mV to light pink at 40 mV). **C.** Mean steady-state activation (converted from the IV relationship seen in **A.**) and inactivation (from the current observed during the final 10 mV step in **B.**) curves for LVGCCs measured in aortic SMCs ($n=8-14$). Smooth curves represent the fit of the data using Eq. 2.4 and 2.5 (activation and inactivation,

respectively). The purple bar highlights the range of V_m that intact SMCs can experience as a result of ChR2(H134R)-mediated depolarisations.

4.2.3 Effect of extended blue light stimulation on whole-cell currents and V_m

As outlined in Chapter 1, arteries require continuous, prolonged exposures to contractile agonists to reach a steady state level of contraction. Thus, the suitability of ChR2(H134R) to maintain depolarising currents and V_m depolarisation for prolonged periods of time (7 min) in aortic SMCs was tested (Fig. 4.5). The steady state photocurrent elicited by 0.08 mW/mm² in SMCs isolated from ChR2(H134R)-SM mice saw a small decrease (~1 pA/pF) in amplitude over the course of 7 minutes. The V_m of isolated aortic SMCs in response to 0.08 mW/mm², a value that caused ~half-maximal depolarisation, remained constantly clamped to ~-27 mV during the 7 min illumination period (Fig. 4.5B). This effect was quantified by measuring the V_m in the first and seventh minute of blue light exposure: no significant change in V_m was detected (Fig. 4.5B).

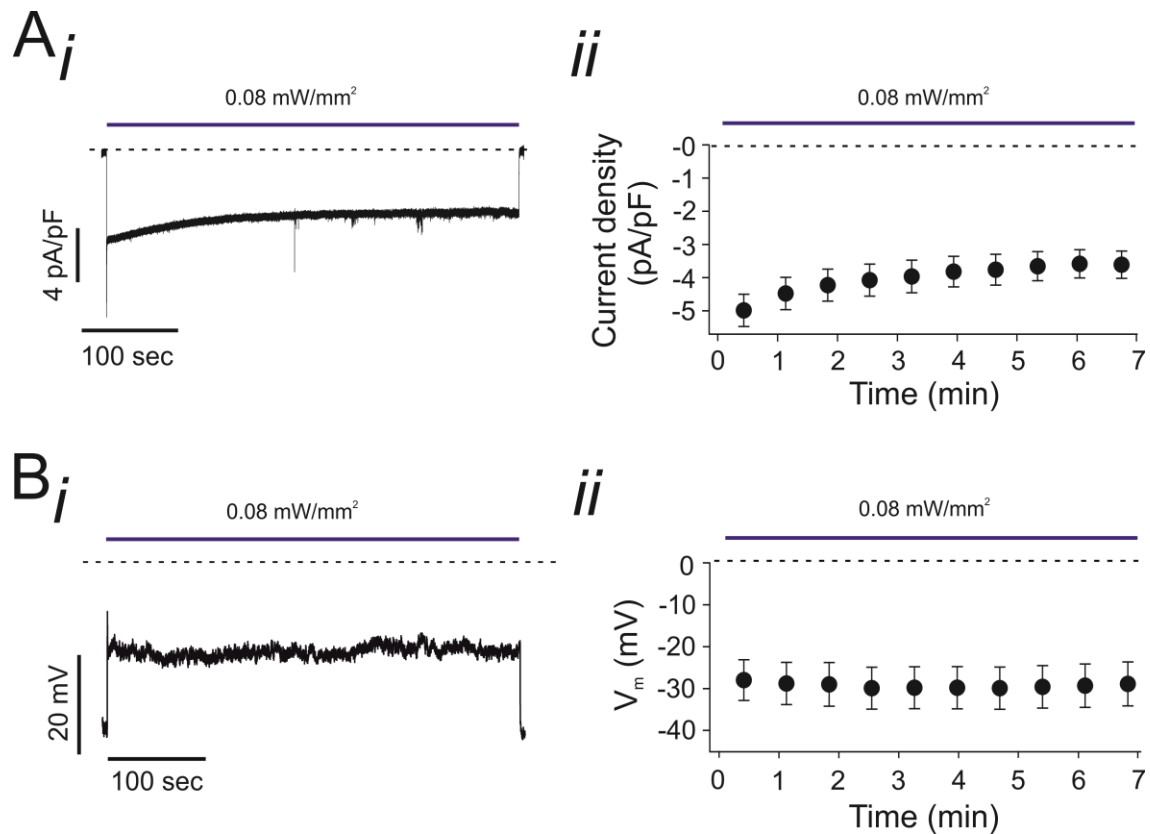


Figure 4.5. Effect of extended blue light stimulation on current and V_m .

Ai. Current recording from an aortic SMC isolated from Chr2(H134R)-SM mouse in response to 7 min blue light stimulation (0.08 mW/mm^2). **Aii.** Mean current density measured over the 7 min exposure period ($n=14$). The V_{hold} was -80 mV . **Bi.** V_m recording of an aortic SMC isolated from Chr2(H134R)-SM mouse in response to 7 min blue light stimulation (0.08 mW/mm^2). **Bii.** Mean V_m measured over the course of the blue light stimulation period (0.08 mW/mm^2) ($n=11$).

4.2.4 Relationship between blue light intensity and tension in isolated artery rings

To investigate whether the histology of the arteries isolated from Chr2(H134R)-SM mice differed from control mice the compliance of the isolated arteries was tested and compared. It was found that artery rings (aortic, mesenteric, pulmonary) obtained from control mice or Chr2(H134R)-SM mice presented the same degree of compliance. This was tested by stretching the artery rings in the absence of light

or contractile agonists (Fig. 4.6). The artery rings were stretched in small increments (50 μm or 100 μm) using the mounting jaws of the myograph (see Chapter 2, Section 2.5.1). This analysis showed that there was no difference in the intrinsic resistance to stretch between the artery rings isolated from the control and the artery rings isolated from the ChR2(H134R)-SM mice. This indicated that, in the dark, ChR2 is completely closed and that it does not provide a source of basal depolarising force. Furthermore, these experiment illustrated that ChR2(H134R)-SM mice have no gross histological alterations in the vessel walls.

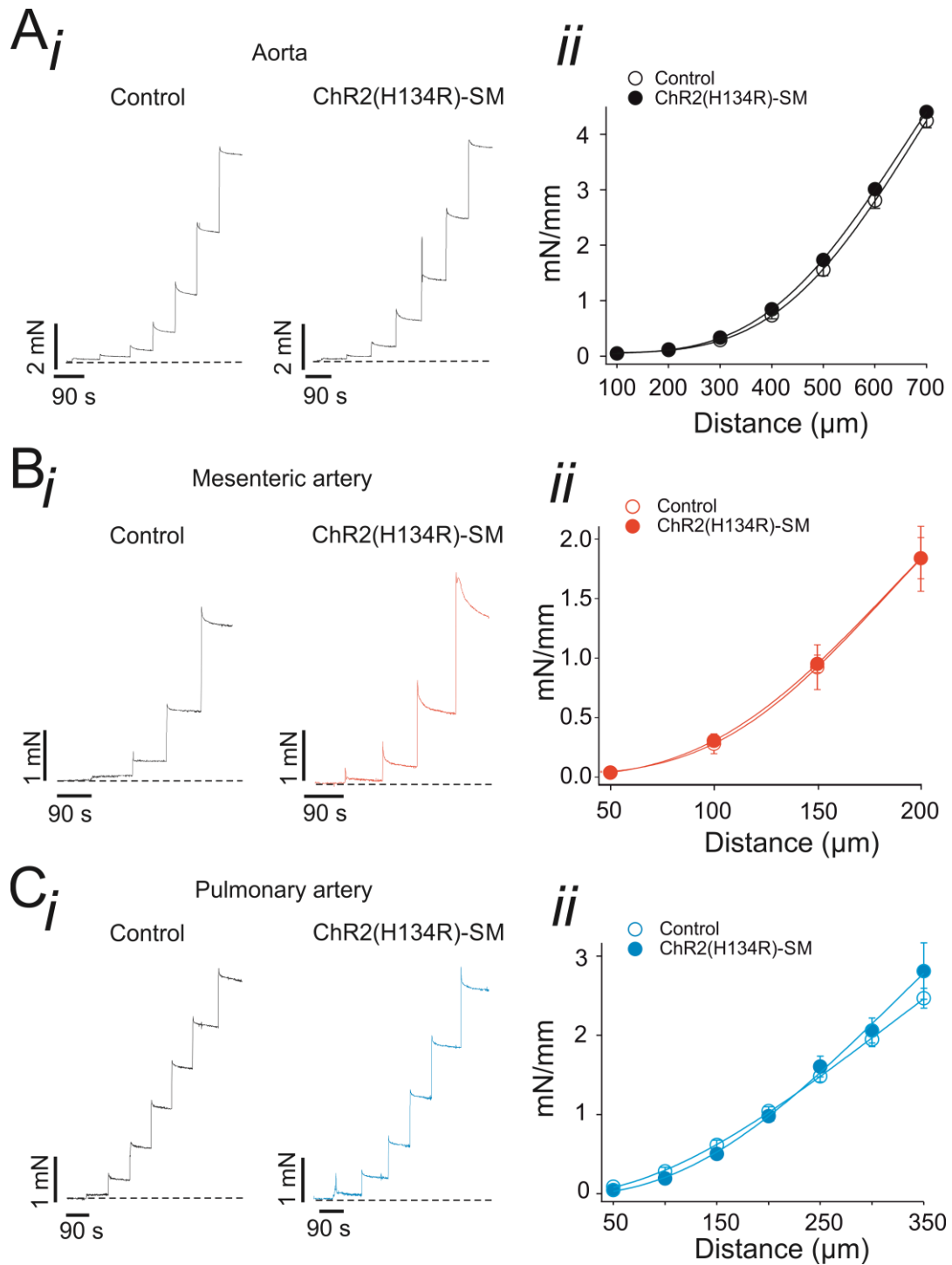


Figure 4.6. Response of aortic, mesenteric and pulmonary artery rings to increasing mechanical stretch.

A_i. Representative change in isometric tension of aortic rings isolated from control or ChR2(H134R)-SM mice in response to incremental increases in stretch. The dashed line represents the baseline tension. **A_{ii}**. Mean isometric tension in aortic rings isolated from control mice ($n=50$) or ChR2(H134R)-SM mice ($n=225$) in response to stretch. **B_i**. Representative change in isometric tension of mesenteric artery rings isolated from control

or ChR2(H134R)-SM mice in response to incremental increases in stretch. The dashed line represents the baseline tension. **Bii.** Mean isometric tension in mesenteric artery rings isolated from control mice (n=5) or ChR2(H134R)-SM mice (n=27) in response to stretch. **Ci.** Representative change in isometric tension of pulmonary artery rings isolated from control or ChR2(H134R)-SM mice in response to incremental increases in stretch. The dashed line represents the baseline tension. **Cii.** Mean isometric tension in pulmonary artery rings isolated from control mice (n=6) or ChR2(H134R)-SM mice (n=21) in response to stretch.

Experiments were then carried out to determine whether artery rings obtained from ChR2(H134R)-SM mice contracted in response to blue light stimulation. To examine the precise relationship between the ChR2(H134R) current amplitude and changes in arterial tone, isometric tension measurements were performed in isolated aortic, mesenteric and pulmonary artery rings obtained from ChR2(H134R)-SM mice. Isolated artery rings were exposed to periods (7 min) of blue light illumination of various intensities (0.02 mW/mm² to 7.2 mW/mm²). The tension of the artery rings increased as the blue light intensity increased (Fig. 4.7). The maximal level of contraction was reached in blue light intensities of >~0.7 mW/mm² in aortic and pulmonary artery rings, whereas the mesenteric artery rings required blue light >~2 mW/mm² to achieve maximal contraction. The fit of the tension *versus* blue light intensity relationships with Eq. 2.7 yielded maximal tension (T_{ss}) of ~3.8 mN/mm, ~0.9 mN/mm and ~1.0 mN/mm for aortic, mesenteric and pulmonary artery rings respectively. The EL_{50} was ~0.1-0.3 mW/mm² and the slope factor (s) was ~1.2-1.5 in each case (Table 4.2). Blue light stimulation produced a very small increase in tension in artery rings obtained from control mice; the contraction measured in the highest blue light intensity stimulation (7.2 mW/mm²) was 0.1±0.1 mN/mm (n=10), 0.2±0.1 mN/mm (n=6) and 0.1±0.1 mN/mm (n=6) for control aortic, mesenteric and pulmonary artery rings, respectively.

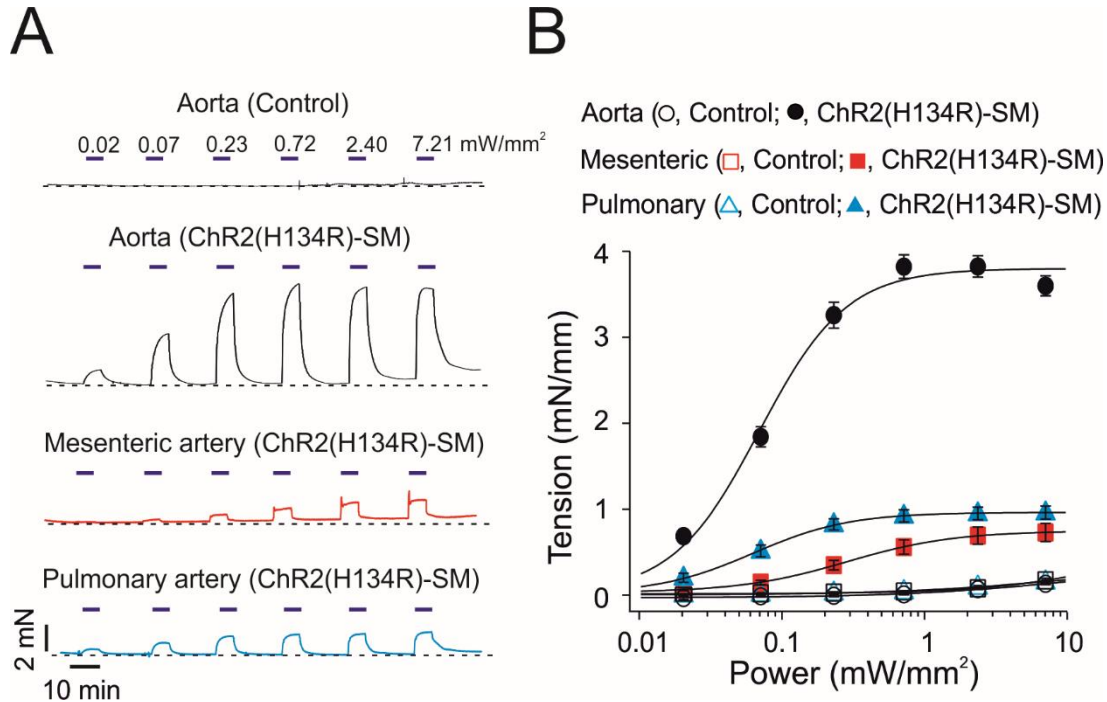


Figure 4.7. Effects of blue light on the tension of isolated artery rings.

A. Representative isometric tension recordings obtained from aortic, mesenteric and pulmonary artery rings isolated from control and ChR2(H134R)-SM mice, as indicated. Artery rings were exposed to periods (7 min) of blue light of various intensities, as indicated by horizontal blue bars. Dashed lines represent baseline tension level. **B.** Mean tension versus blue light intensity relationships for artery rings obtained from control ($n=6-10$) or ChR2(H134R)-SM mice ($n=8-36$), as indicated. The curves through open symbols (control) were drawn by eye (error bars within the symbols). The response of the different artery types isolated from control mice overlap. The curves through the filled symbols represent the best fit of the data with Eq. 2.7.

Table 4.2. Parameters for the tension versus blue light intensity relationships for artery rings obtained from ChR2(H134R)-SM mice.

Parameter	Aorta	Mesenteric artery	Pulmonary artery
EL_{50} (mW/mm ²)	0.09±0.01($n=36$)	0.30±0.05 ($n=14$)	0.07±0.01 ($n=8$)
s	1.5±0.1 ($n=36$)	1.1±0.1 ($n=14$)	1.4±0.1 ($n=8$)
T_{ss} (mN/mm)	3.8±0.1 ($n=36$)	0.9±0.1 ($n=14$)	1.0±0.1 ($n=8$)

Parameters (EL_{50} , s and T_{max}) obtained from the Hill fit (Eq. 2.7) of the tension versus blue light intensity relationship for aortic, mesenteric and pulmonary artery rings obtained from ChR2(H134R)-SM mice.

4.2.5 Relationship between the extracellular KCl concentration and tension in isolated artery rings

Next, the blue light induced contraction was compared with the contraction triggered by elevation of extracellular KCl concentration ($[KCl]_e$) up to 100 mM. The maximal asymptotic degree of blue light-induced contraction, as extrapolated from the Hill fit (Eq. 2.7), for aortic, mesenteric and pulmonary artery rings obtained from ChR2(H134R)-SM mice was ~100%, ~80% and ~105% of the maximal asymptotic tension obtained in response to high $[KCl]_e$ (Fig. 4.8 and Table 4.3). Thus, all artery types tested experienced maximal or near-maximal tension as a result of maximal ChR2(H134R) opening.

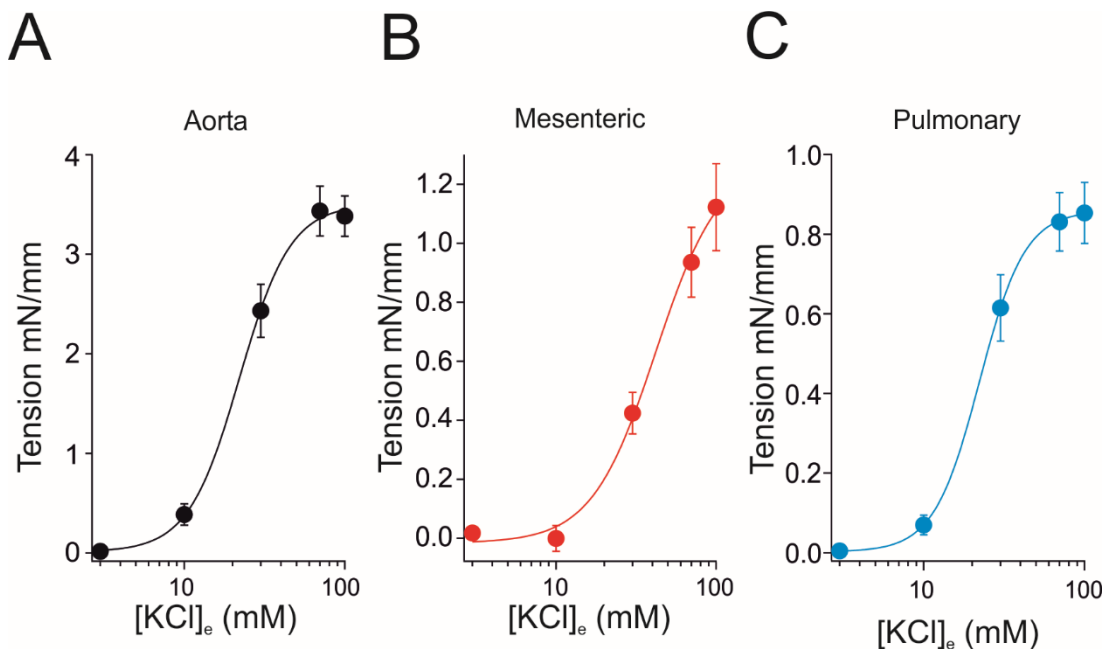


Figure 4.8. Effects of $[KCl]_e$ on the tension of rings of various artery types isolated from ChR2(H134R)-SM mice.

Mean tension versus $[KCl]_e$ relationships for the different artery types (aortic, **A**, mesenteric, **B**, and pulmonary, **C**) (n=8-19). The curves through the filled symbols represent the best fit of the data with Eq. 2.7.

Table 4.3. Parameters for tension versus $[KCl]_e$ relationships for artery rings obtained from ChR2(H134R)-SM mice

Parameter	Aorta	Mesenteric artery	Pulmonary artery
EC_{50} (mM)	24.0±1.4 (n=19)	44.6±6.0 (n=8)	22.9±1.8 (n=8)
s	3.1± 0.1 (n=19)	2.5±0.2 (n=8)	3.2±0.2 (n=8)
T_{ss} (mN/mm)	3.5±0.2 (n=19)	1.3±0.2 (n=8)	0.9±0.1 (n=8)

Parameters (EC_{50} , s and T_{ss}) extrapolated from the Hill fit (Eq. 2.7) of the tension versus $[KCl]_e$ relationship for aortic, mesenteric and pulmonary artery rings obtained from ChR2(H134R)-SM mice.

4.2.6 Relationship between duration of blue light or elevated $[KCl]_e$ exposure and tension of isolated artery rings

Experiments shown in Figure 4.6 illustrate that prolonged exposure (7 min) of blue light to isolated artery rings led to a stable contraction. Two possible underlying scenarios in terms of blue light induced contraction were therefore tested. These were: i) short pulses of blue light initiated contraction of isolated artery rings or ii) the blue light stimulation had to be maintained to achieve steady-state contraction. Aortic rings isolated from ChR2(H134R)-SM mice were exposed to light of 0.23 mW/mm² for various time periods (Fig. 4.9A). This light intensity was expected to shift the V_m to ~-25 mV, based on the current-clamp recordings in isolated aortic SMCs described above (Fig. 4.3). The tension of ChR2(H134R)-SM aortic rings

increased rapidly in response to the light stimulation, but removal of the stimulus resulted in an immediate start of relaxation. Light of 0.23 mW/mm^2 applied for 2 s, 1 min or 7 min resulted in an increase in tension of $0.6 \pm 0.1 \text{ mN/mm}$ ($n=6$), $1.6 \pm 0.2 \text{ mN/mm}$ ($n=6$) and $2.7 \pm 0.3 \text{ mN/mm}$ ($n=6$), respectively (Fig. 4.9B).

To determine if the ChR2(H134R)-mediated contraction was directly linked to V_m depolarisation, the extent of contraction caused by ChR2(H134R) opening was compared with the contraction caused by elevated $[\text{KCl}]_e$. PSS was supplemented with 50 mM KCl, as this was expected to produce V_m depolarisation to $\sim -25 \text{ mV}$ as calculated using Eq. 1.7 with the values for p_{Cl} , p_{K} , p_{Ca} and $[\text{ion}]_i$ and $[\text{ion}]_e$ reported in Table 1.1. Aortic rings were exposed to elevated $[\text{KCl}]_e$ for 1 min or 7 min. Note that exchange of solution in the myography chamber required $\sim 1 \text{ s}$, thus KCl application for 2 s was not attempted as the exchange of solution during this time interval could not be accurately controlled. Application of 50 mM KCl for 1 min or 7 min led to an increase in artery tension to $1.9 \pm 0.1 \text{ mN/mm}$ ($n=6$) and $2.9 \pm 0.2 \text{ mN/mm}$ ($n=6$), respectively. Wash out with PSS resulted in rapid relaxation. In addition to the similar levels of contraction achieved, the time-course of blue light-induced and KCl-induced contractions were very similar and each followed a double exponential time-course with a fast (τ_f) and slow (τ_s) component. These components were well quantifiable during the long (7 min) stimulations. KCl stimulation was associated with τ_f of $6.5 \pm 1.3 \text{ s}$ ($n=6$) and a τ_s of $148 \pm 11 \text{ s}$ ($n=6$), whereas light stimulation was characterised by τ_f of $3.6 \pm 0.6 \text{ s}$ ($n=6$) and τ_s of $139 \pm 32 \text{ s}$ ($n=6$). These experiments showed that, in similarity with the well-established contractile effect of elevated $[\text{KCl}]_e$, blue light stimulation needed to be continuously maintained to sustain aortic smooth muscle contraction.

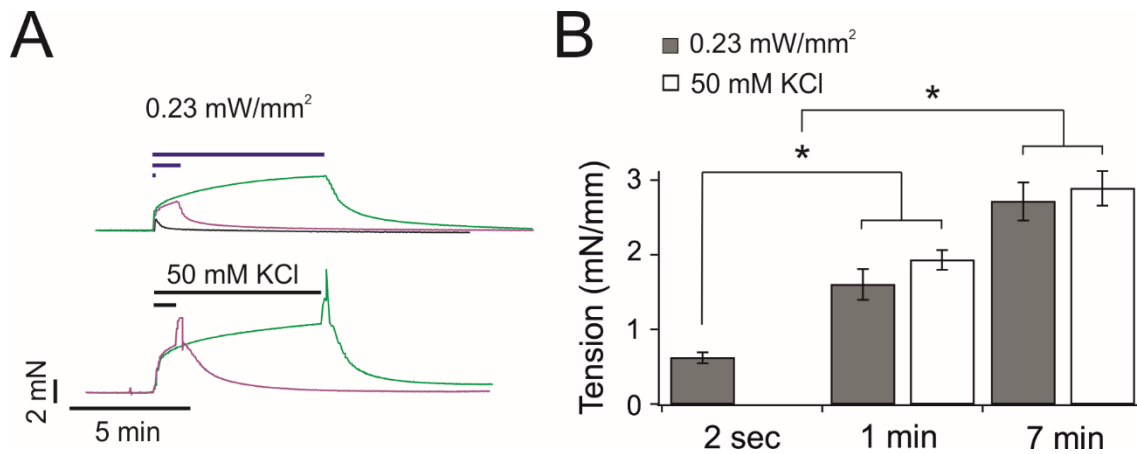


Figure 4.9. Comparison of blue light induced and KCl induced contraction.

A. Isometric tension recordings obtained from isolated aortic rings obtained from ChR2(H134R)-SM mice in response to periods of exposure to blue light (0.23 mW/mm^2) of different durations (2 s, 1 min and 7 min), or periods of exposure (1 min or 7 min) to PSS supplemented with 50 mM KCl, as indicated by the horizontal bars. Traces are represented in different colours to ease visual inspection. **B.** Mean tension developed by isolated aortic rings obtained from ChR2(H134R)-SM mice in response to 0.23 mW/mm^2 blue light or 50 mM KCl for periods of different duration ($n=6$ in each case). *, $P<0.05$ (One-Way ANOVA).

4.2.7 Effects of light in the presence of PE

The isolated artery ring experiments described above were performed in the absence of contractile agonists. In the presence of contractile agents, such as PE, isolated wild-type arterial smooth muscle have been reported to relax in response to blue light due to melanopsin signalling [358,359,361]. The next set of experiments aimed to test whether ChR2(H134R)-mediated depolarisation and contraction can overcome the relaxing response of arteries to blue light in the presence of PE. First, the relaxation of aortic rings obtained from control mice in response to blue light was quantified. In the presence of PE (1 μM) aortic rings relaxed in a blue light intensity dependent manner (Fig. 4.10A). For example, blue light intensity of 0.72 mW/mm^2 resulted in a ~ 0.3 -fold reduction in artery ring tension (Fig. 4.10B). Fit of

the tension versus light intensity relationship with Eq. 2.8 yielded an EL_{50} of 0.1 ± 0.1 mW/mm^2 ($n=7$), a slope factor (s_n) of 1.9 ± 0.2 ($n=7$) and a maximal normalised steady state tension (T_{SSn}) of 0.7 ± 0.1 ($n=7$).

To establish whether opening of ChR2(H134R) was sufficient to counteract the relaxation described above, aortic rings isolated from ChR2(H134R)-SM mice were exposed to blue light of increasing intensities in the presence of PE ($1 \mu M$). Under these conditions, aortic rings contracted in response to blue light (Fig. 4.10A). For example, blue light of $0.72 mW/mm^2$ elicited a ~4-fold increase in tension (Fig. 4.10B). The fit of the tension versus blue light intensity relationship with Eq. 2.8 yielded an EL_{50} of $0.1 \pm 0.1 mW/mm^2$ ($n=6$), a slope factor (s_n) of 1.1 ± 0.1 ($n=6$) and a T_{SSn} of 4.6 ± 0.7 ($n=6$).

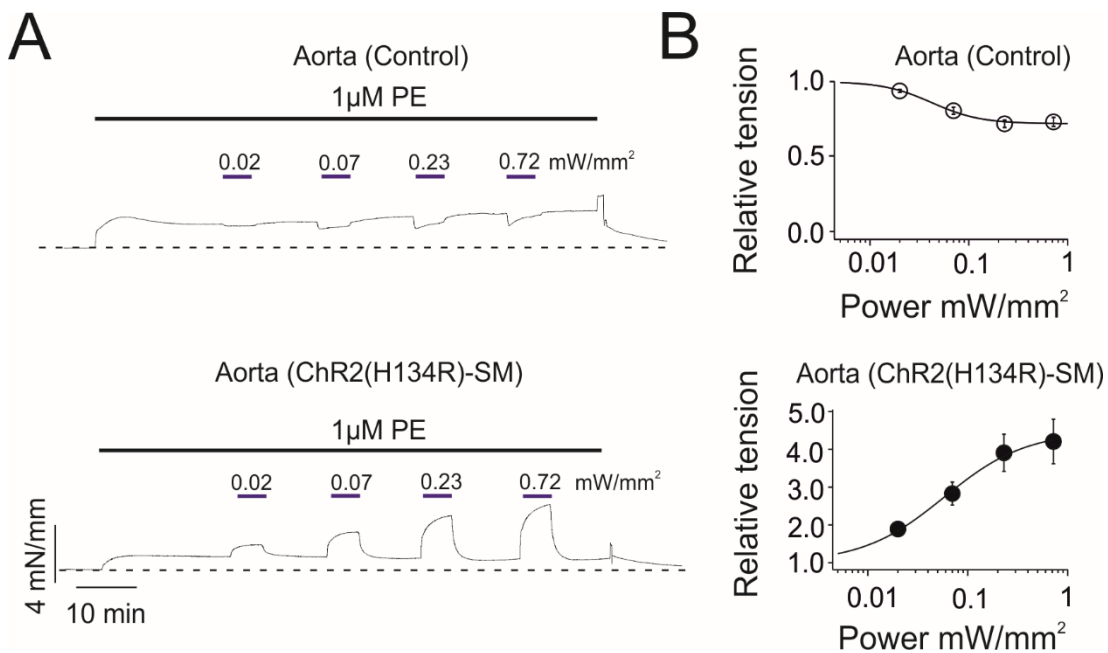


Figure 4.10. Effect of blue light on tension of isolated aortic rings in the presence of PE.

A. Isometric tension recordings obtained from aortic rings isolated from control or ChR2(H134R)-SM mice, as indicated. Artery rings were exposed to PE ($1 \mu M$) and blue light as indicated by the black and blue horizontal bars, respectively. The dashed lines indicate the basal tension level. **B.** Mean tension versus blue light intensity relationships for

aortic rings obtained from control or ChR2(H134R)-SM mice, as indicated. The change in tension elicited by blue light was expressed relative to the tension measured in the presence of PE (n=6-7). The smooth curves through symbols represent the best fit of the data with Eq. 2.8.

4.3 Discussion

The main findings of this chapter were:

- 1) Blue light activation of ChR2(H134R) expressed selectively in SMCs results in inward, depolarising currents leading to artery ring contraction very similar to that induced by elevated $[KCl]_e$. This is likely to be as a result of activating endogenous LVGCCs following depolarisation of the SMCs.
- 2) Current amplitude, degree of V_m depolarisation, and artery ring contraction can be finely controlled by altering the intensity of the stimulatory light.
- 3) Native relaxation of artery rings previously described in the literature was overcome by the contraction induced by ChR2(H134R) mediated currents.
- 4) ChR2(H134R) can be activated for extended periods of time providing a depolarising current at a similar level throughout the stimulation period.

This experimental analysis reveals the ChR2(H134R)-SM mice as a powerful experimental model to selectively control the excitability of SMCs and the tone of isolated arteries.

4.3.1 ChR2(H134R) expression and current amplitude in isolated SMCs

The level of ChR2(H134R) expression in vascular SMCs was pulmonary artery>aorta>mesenteric artery. The amplitude of the current density also followed this order. Possible causes of unequal ChR2(H134R) protein expression in different

SMC types include: i) variable levels of transcription factors acting on the transgelin promoter, which in turn controls the expression of the Cre recombinase and/or ii) differences in how proteins are trafficked and expressed in different SMCs types. Regardless of the underlying causes of variable expression of ChR2(H134R) in different artery types, the degree of expression in the arteries tested was sufficient to promote maximal or near-maximal contraction in all artery types tested. The difference in current amplitude may also be in part due to ChR2(H134R) undergoing varying degrees of post-translational modifications in different cell types [417]. Interestingly, the EL_{50} of the blue light induced current was similar ($\sim 0.3 \text{ mW/mm}^2$) in SMCs isolated from the various artery types. As evidenced in the above results the current could be maintained for extended periods of time; the small decrease in current amplitude seen over the $\sim$ first minute could be as a consequence of Ca^{2+} and Na^+ accumulation that can affect the E_{rev} as previously described in relation to K^+ currents [418]. However, this observation does not translate to a variation in current-clamp patch clamp recordings.

Mesenteric artery rings exhibited a greater EL_{50} than aortic and pulmonary artery rings. To examine if was due to a reduced level of ChR2(H134R) expression the contractile response to blue light and elevated $[\text{KCl}]_e$ was compared. The response to varying concentrations of extracellular KCl plateaued for both the aortic and pulmonary artery rings at similar levels of tension to that seen in response to blue light stimulation. However, the mesenteric artery rings produced greater level of contraction in response to very high (70-100 mM) KCl than to the maximal optical stimulation. This is evidence that the reduced expression of ChR2(H134R) in mesenteric arteries has implications for vascular optogenetics; variation in the extent of blue light induced contraction in response to specific intensities between

vessel types could potentially result in the degree of contraction deviating from the intended one. This variation could be particularly important when using vascular optogenetics to reduce the blood flow to specific tissues for experimental purposes. Thus, the ChR2(H134R) expression level and sensitivity to blue light stimulation needs to be adequately determined for the use of vascular optogenetics to control the tone of different artery types.

The difference in temperature between the electrophysiological recordings (~22 °C) and the myography experiments (~37 °C) could potentially have an impact on the free $[Ca^{2+}]_i$ or the kinetics of ChR2(H134R). However, as the buffering capacity for Ca^{2+} is rather constant within this range (estimated free $[Ca^{2+}]_i$ ~40 nM and ~30 nM at 22 °C and 37 °C, respectively (using MAXCHELATOR)), I do not anticipate a significant impact. In the intact vessel, the Ca^{2+} buffering mechanisms are not the same as in the isolated SMCs used for electrophysiological recordings as the intracellular solution cannot be replaced in the intact vessel. However, it has previously been established that EGTA can mimic native Ca^{2+} buffers in the 1-10 mM range [419]. Thus, for purposes of this discussion I assume that the intracellular buffering of SMCs in the intact vessel behaves similarly to EGTA in the pipette solution used for patch-clamp experiment. The impact of the temperature sensitivity of ChR2(H134R) can be assumed to be minimal as the temperature change primarily affect the fast kinetic of the channel activation and inactivation (that occur within ~100 ms) and has temperature coefficient (Q_{10}) of 2-3 [420], whereas the majority of the experiments were carried out on a much longer time scale (minutes).

4.3.2 *Activation of ChR2(H134R) results in V_m depolarisation and LVGCC opening in isolated SMCs*

Application of blue light stimulus led to reversible V_m depolarisation, reaching ~ -20 mV. This is less depolarised than what would be expected if ChR2(H134R) was the only channel in the membrane. It is possible that Ca^{2+} influx through ChR2(H134R) or via LVGCCs, which are activated by V_m depolarisation triggered by ChR2(H134R), resulted in activation of BK_{Ca} channels. This would antagonise the depolarising effect of ChR2(H134R) and explain why the max depolarisation caused by ChR2(H134R) is still below the ~ 0 mV as expected for a non-selective cation channel.

LVGCCs are a principal and well established route of Ca^{2+} influx in vascular SMCs [165,421]. In isolated aortic SMCs, LVGCC window currents spanned from approximately -40 mV to 10 mV, which compares to previous studies [422,423]. Window currents represent a range of V_m in which the activation and inactivation curves overlap [110]. Within this range the channels are activated but not inactivated. This indicated that a small light-dependent depolarisation from the resting V_m (~ -40 mV under the experimental conditions used in this study) was sufficient to bring the V_m into a range where LVGCCs were active. Therefore, ChR2(H134R) primarily served as a switch for LVGCC activation.

The mean resting potential of the isolated SMCs is within the range reported in the literature [115,128–130]. However, in intact vessels the V_m , assessed using microelectrodes, is more hyperpolarised (~ -50 mV to -60 mV [89,97,122,131–133]). This discrepancy could be due to the cell isolation process, which involves trypsin, that may damage membrane transport mechanism and/or cause a small degree of “leak” current. Alternatively, the somewhat slightly depolarised state of

isolated cells could be the results of a loss of EC-derived hyperpolarising factors (e.g. NO). The current measurements of the V_m could be complemented with sharp electrode recordings of the V_m in an intact artery (e.g. [89]). Nevertheless, the V_m reported here is within the previously reported range and adequately shows the cross-talk between the V_m and the LVGCC activity.

4.3.3 Basal artery tone and response to blue light stimulus or elevated $[KCl]_e$

As evidenced by the analysis of the stretch of the artery rings isolated from Chr2(H134R)-SM mice and control mice, the expression of Chr2(H134R) did not affect the intrinsic resistance of the artery rings to stretch (vessel compliance). This was not a foregone conclusion as even the light in the laboratory could have caused a small amount of Chr2(H134R) activation and thus a small level of added basal contraction.

In aortic, mesenteric and pulmonary artery rings isolated from Chr2(H134R)-SM mice contracted in response to blue light stimulation; higher light intensities elicited greater levels of contraction. Combining this data with the V_m and LVGCC recordings, one can infer that increasing blue light intensity led to greater depolarising currents, shifting the V_m into a range where the likelihood of LVGCC activation was more likely thus providing a greater source of Ca^{2+} influx and a greater activation of the contractile machinery of the SMC. Blue light stimulus was required to be maintained for multiple minutes before a steady state was achieved.

Up until 0.7 mW/mm^2 the contraction is completely reversible, with a complete return to baseline within 5 min. At higher light intensities, however, a complete return, when possible, took a longer period of time. At the 2.4 and 7.2 mW/mm^2 there was an incomplete return. The lack of recovery could be due to some unknown damaging

effect which in turn would lead to loss of normal physiological function possibly similar to neuronal excitotoxicity involving Ca^{2+} accumulation (as seen in isolated rat granule cells [424]), or a change in the pH_i of the SMCs due to H^+ flux through ChR2 as observed in mouse glial cells [324].

The time course of the change in tension of aortic rings in response to blue light or elevated $[\text{KCl}]_e$ was remarkably similar. Furthermore, continuous application of either stimuli was necessary for the tension of isolated aortic rings to reach a maximal, steady-state value. These observations suggest that ChR2(H134R) acted as a depolarising mechanism (providing an “optical voltage-clamp” method) in a similar way to high $[\text{KCl}]_e$ (constituting “chemical voltage-clamp”). Vascular optogenetics provides significant advantages compared to depolarisation achieved by elevation of $[\text{KCl}]_e$. For instance, elevated KCl could not be applied selectively to SMCs without affecting neighbouring cells and tissues. In contrast, optogenetics can enable selective control of SMC excitability. Furthermore, elevation of extracellular K^+ concentration may affect transport mechanisms such as the Na^+/K^+ ATPase, which could lead to an alteration in the intracellular Na^+ concentration. In turn, this may affect other secondary transport mechanisms as well as the action of numerous enzymes and intracellular proteins, as reviewed in [425].

4.3.4 ChR2(H134R) activation overcomes native relaxation of isolated arteries

Previous published studies showed that systemic arteries obtained from wild-type animals pre-constricted with NA or PE, relaxed in response to blue light [358,359,361]. A key mechanism involved in this response was the activation of melanopsin receptors [361]. Dilation in response to blue light has also been observed in murine cerebral arterioles, although the molecular mechanism and signalling pathway involved in photodilation in this circulation remains unclear [360].

It was determined that ChR2(H134R)-mediated contraction could overcome the native relaxation in murine conduit arteries in response to blue light. This is a prerequisite for the use of vascular optogenetic *in vivo* as the arteries are exposed to varying levels of circulating or locally released catecholamines, depending on the extent of sympathetic stimulation. This knowledge indicates a possible use of vascular optogenetics *in vivo* as a tool to control vessel tone.

4.3.5 *The impact of potential tonic activation of ChR2(H134R) in living mice*

The current study did not involve blood pressure measurements in the ChR2(H134R)-SM or control mice. It is possible that tonic activation of ChR2(H134R) by ambient light could result in a change in the resting blood pressure of the ChR2(H134R)-SM mice. However, as the light intensity of the main lights in the animal holding facility is below the threshold required for activation of ChR2(H134R) in HEK-293T cells and SMCs ($<0.001 \text{ mW/mm}^2$) it could be assumed that an effect, if any, would be very small. The low penetrance of blue light through skin makes a significant effect less likely [258,426]. Attenuation of light through tissues is due to absorption by tissue chromophores and light scattering by the tissue. The extent of light attenuation has been well defined in brain tissues where intensity of blue light diminishes by $>95 \%$ across 1 mm [426]. Similar lack of penetrance can be expected in skin and muscle as the blue wavelength of light is readily absorbed. Furthermore, the presence of fur would likely further reduce the amount of light reaching the skin. However, blood vessels in areas not covered by fur (tail, ears and the retina) could be affected. Compromised blood flow to the retina, as a consequence of contraction due to ChR2(H134R) stimulation by ambient light, could potentially lead to impaired vision. The vision of the mice could be tested as outlined in [324]. Alternatively, the blood flow could be directly

measured using confocal fluorescence microscopy [427]. If the vision of Chr2(H1314R)-SM mice was found to be impaired, it might have animal welfare implications for the use of these mice in future studies. However, as mice are nocturnal, the impact of any stimulation by the animal holding facility lights could be assumed to be limited.

4.3.6 Conclusion

The work presented in this chapter has established that the expression of Chr2(H134R) in SMCs enabled control of vessel tone in response to blue light stimulation. The blue light induced contraction is caused by inward currents that cause depolarisation of the plasma membrane that is sufficient to shift the V_m into a range where LVGCCs are activated. From this, it can be suggested that Chr2(H134R) acts as a switch for endogenous contractile mechanisms. The similarities in the kinetics and degree of contraction induced by blue light stimulation and elevated $[KCl]_e$ suggests that optogenetically induced contraction acts via similar pathways as elevated $[KCl]_e$. The focus for the next chapter is to define the precise ionic mechanism linking Chr2(H134R) activation to SMC contraction.

Chapter 5

Investigating the ionic mechanism
of vascular optogenetics

5.1 **Introduction**

The data presented in Chapter 4 demonstrated that expression of ChR2(H134R) in SMCs constitutes a depolarising mechanism which led to V_m depolarisation, activation of LVGCCs, and artery contraction. The focus of this chapter was to define the exact complement of endogenous ionic mechanisms recruited during ChR2(H134R)-mediated artery contraction.

5.2 **Results**

5.2.1 *Role of LVGCCs in ChR2(H134R)-induced artery contraction*

To determine whether blue light induced contraction required influx of Ca^{2+} from the extracellular solution, aortic rings obtained from ChR2(H134R)-SM mice were exposed to blue light in Ca^{2+} -free physiological salt solution (PSS) while the isometric vessel tension was recorded. Under these conditions, blue light stimulation produced a very small contraction. For example, the tension measured during 7.2 mW/mm^2 blue light stimulation was $0.1 \pm 0.1 \text{ mN/mm}$ ($n=7$) compared to $3.7 \pm 0.1 \text{ mN/mm}$ ($n=36$) in the presence of extracellular Ca^{2+} (Fig. 5.1).

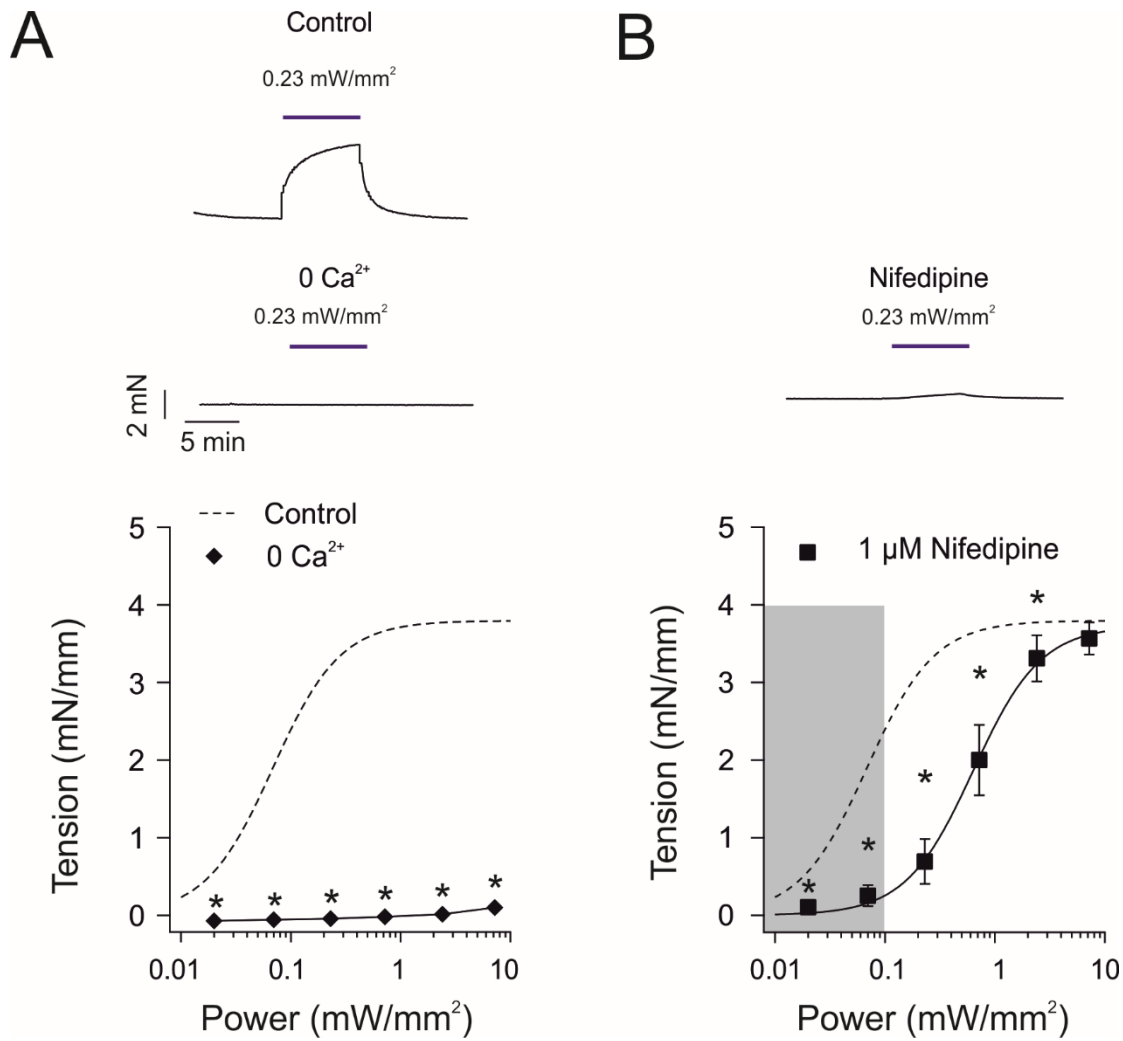


Figure 5.1. Effect of LVGGC modulation and omission of extracellular Ca²⁺ on blue light-induced tension of aortic rings obtained from Chr2(H134R)-SM mice.

A. Top panels represent isometric tension recordings obtained from aortic rings isolated from Chr2(H134R)-SM mice in response to 0.23 mW/mm² blue light in PSS (control) or a nominally Ca²⁺-free PSS (0 Ca²⁺). The lower panel represents mean tension versus blue light intensity relationship for experiments conducted in Ca²⁺-free PSS (n=7). The dashed sigmoidal curve represents the Hill fit (Eq. 2.7) of the aortic ring tension versus blue light intensity relationship presented in Fig. 4.4B in Chapter 4. **B.** Top panel represents isometric tension recordings obtained from aortic rings isolated from Chr2(H134R)-SM mice in response to 0.23 mW/mm² blue light in the presence of PSS supplemented with nifedipine (1 μM). The lower panel represents mean tension versus blue light intensity relationship for experiments conducted in PSS supplemented with 1 μM nifedipine (n=8). The grey bar indicates the range of blue light intensities that result in contraction in a fully LVGGC dependent fashion. Continuous sigmoidal curve through the square symbols indicates the

best fit of the data with Eq. 2.7. Dashed sigmoidal curve is the Hill fit (Eq. 2.7) of the aortic ring tension versus blue light intensity relationship presented in Fig. 4.4B in Chapter 4.

The contraction of isolated aortic rings was dramatically reduced by application of the LVGCC blocker nifedipine at a concentration causing full blockade of LVGCCs ($1\ \mu\text{M}$) (Fig. 5.1B) [428]. Nifedipine shifted the EL_{50} of the tension versus blue light intensity relationship fitted with Eq. 2.7 from $\sim 0.1\ \text{mW}/\text{mm}^2$ to $\sim 0.8\ \text{mW}/\text{mm}^2$, and the slope factor (s) slightly increased from ~ 1.5 to ~ 2 , while the maximal tension achieved (T_{ss}) remained unaltered ($\sim 3.7\ \text{mN}/\text{mm}$) (Table 5.1).

Table 5.1. Parameters for the tension versus blue light relationships for aortic rings obtained from Chr2(H134R)-SM mice in the presence of nifedipine

Parameter	Mean values
$EL_{50}\ (\text{mW}/\text{mm}^2)$	$0.77 \pm 0.18\ (n=8)^*$
s	$2.0 \pm 0.2\ (n=8)^*$
$T_{ss}\ (\text{mN}/\text{mm})$	$3.7 \pm 0.2\ (n=8)$

Parameters (EL_{50} , s and T_{ss}) extrapolated from the Hill fit (Eq. 2.7) of the tension versus blue light intensity relationship for aortic rings obtained from Chr2(H134R)-SM mice in the presence of $1\ \mu\text{M}$ nifedipine. *, Statistical significance against experiment conducted in the absence of nifedipine and reported in Table 4.1 ($P < 0.05$, t-test).

5.2.2 Effect of nifedipine on LVGCC currents

Nifedipine is a light-sensitive compound that photodegrades into a 2-nitroso derivative when exposed to light [428]. Therefore, the possibility that nifedipine was degraded during exposure to blue light was examined via a separate set of functional electrophysiological experiments. The extracellular whole-cell patch-clamp solution containing nifedipine ($1\ \mu\text{M}$) was illuminated with high-intensity blue light ($2.4\ \text{mW}/\text{mm}^2$) for 10 min. The extent to which LVGCC current in isolated aortic

SMCs was inhibited by this solution was indistinguishable from that caused by a nifedipine-containing solution that was not exposed to light (Fig. 5.2). Thus, exposure of nifedipine to blue light appeared to have no effect on its inhibitory effect on LVGCCs in our experiments.

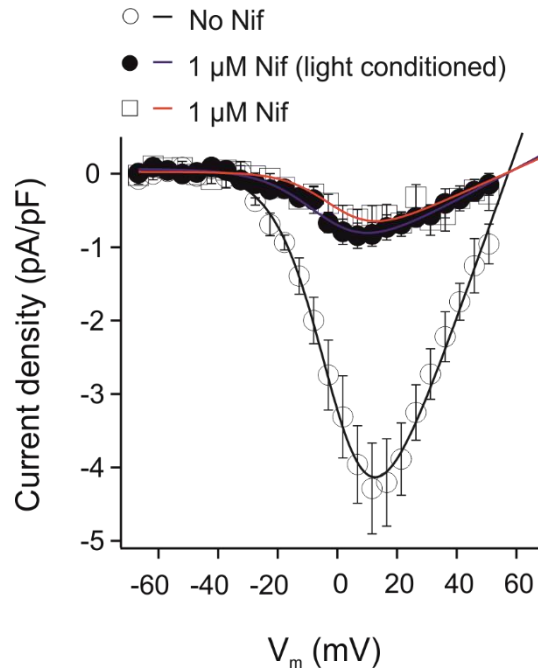


Figure 5.2. LVGCC currents.

Mean whole-cell LVGCC currents versus V_m relationships in the absence ($n=14$) or presence of $1 \mu\text{M}$ nifedipine (Nif) which was either kept in the dark ($n=7$) or exposed to blue light (2.4 mW/mm^2) for 10 min (light conditioned) ($n=12$), as indicated. Curves through symbols represent the best fit of the data with Eq. 2.4.

The isometric tension recordings shown in Figure 5.1 demonstrated that, in response to light stimulations below $\sim 0.1 \text{ mW/mm}^2$, aortic ring contraction required LVGCC activation as nifedipine fully prevents blue light induced contraction at these intensities. Presumably contractions triggered by blue light stimulations of greater intensity would involve, at least in part, Ca^{2+} influx through ChR2(H134R) and would result in a level of contraction which may not be reached under normal physiological

conditions. To test this possibility, the effects of nifedipine was tested on aortic rings obtained from control mice in response to increased $[KCl]_e$, to induce depolarisation (Fig. 5.3). Nifedipine ($1\ \mu M$) almost completely abolished the KCl induced contraction of aortic rings (Fig. 5.3A). For example, $70\ mM\ [KCl]_e$ induced contraction of $0.2 \pm 0.1\ mN/mm$ in the presence of nifedipine compared to $\sim 3.5\ mN/mm$ in the absence (Fig. 5.3B). These results were consistent with the idea that activation of ChR2(H134R) by high intensity blue light may produce Ca^{2+} influx directly into the SMCs through ChR2(H134R).

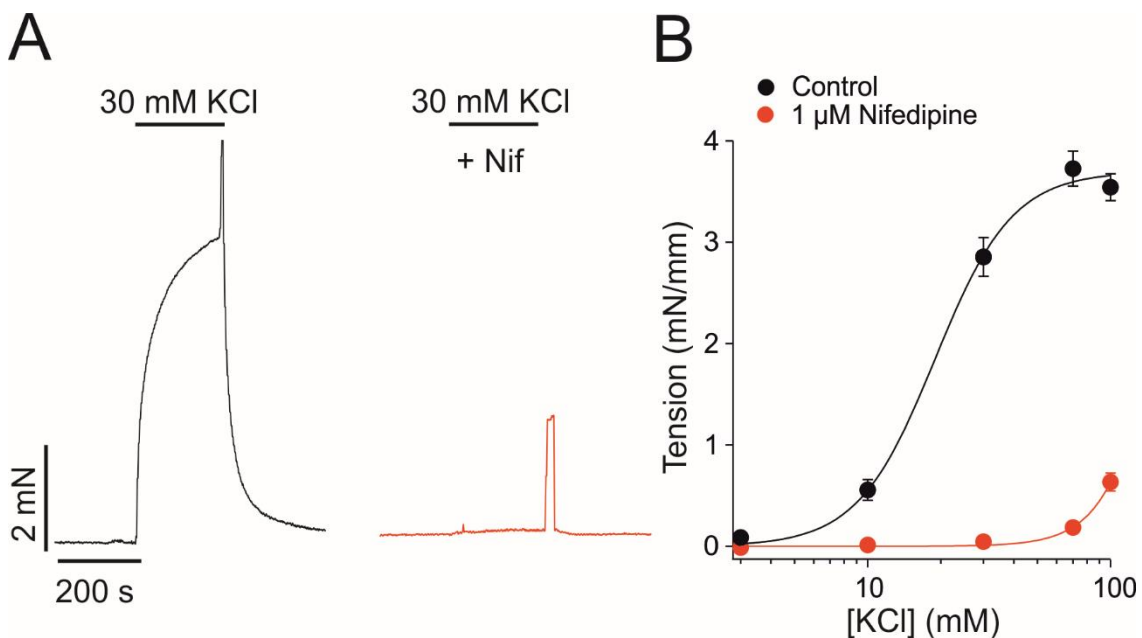


Figure 5.3. Effect of nifedipine on elevated $[KCl]_e$ induced contraction of aortic rings obtained from control mice.

A. Representative recordings of isometric vessel tension in response to elevated $[KCl]_e$ in the absence (left) or presence (right) of $1\ \mu M$ nifedipine. **B.** The mean tension normalised for vessel length versus $[KCl]_e$ relationship for experiments conducted in the presence ($n=5$) or absence ($n=32$) of $1\ \mu M$ nifedipine as indicated. The data in the absence of nifedipine is replotted from Fig. 4.5A in Chapter 4. Continuous sigmoidal curve through the symbols indicates the best fit of the data with Eq. 2.6.

5.2.3 Effect of NA on artery tone

To evaluate the maximal degree of tension that the artery may develop *in vivo*, the response of isolated aortic artery rings to the physiological agonist NA was examined. The maximal contraction achieved by aortic artery rings in response to NA was ~2.3 mN/mm (Fig. 5.4 and Table 5.2). This corresponds to the contraction mediated by ChR2(H134R) in response to blue light of ~0.1 mW/mm². Thus, light stimulations <~0.1 mW/mm² were considered those that elicit a contraction that resemble, in terms of intensity and underlying Ca²⁺ handling mechanisms, physiological contractions.

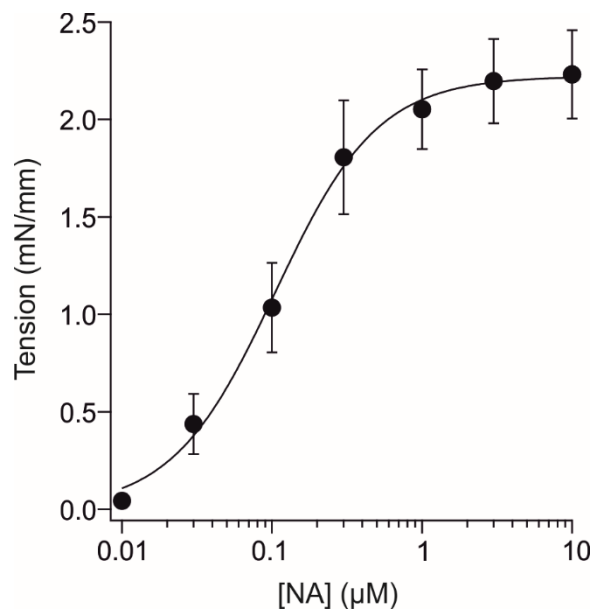


Figure 5.4. Effects of NA on the tension of aortic rings isolated from ChR2(H134R)-SM mice.

Mean tension versus NA concentration ($[NA]$) relationship ($n=6$). The curve through the symbols represents the best fit of the data with Eq. 2.6.

Table 5.2. Parameters for tension versus [NA] relationships for artery rings obtained from ChR2(H134R)-SM mice.

Parameter	Mean value
EC_{50} (mM)	0.13 ± 0.03 (n=6)
s	1.48 ± 0.24 (n=6)
T_{ss} (mN/mm)	2.27 ± 0.29 (n=6)

Parameters (EC_{50} , s and T_{ss}) obtained from the Hill fit (Eq. 2.6) of the tension versus [NA] relationships for aortic rings obtained from ChR2(H134R)-SM mice.

5.2.4 Contribution of Ca^{2+} -elevating mechanisms to blue light induced contraction

To determine the contribution of ryanodine receptor mediated Ca^{2+} -induced Ca^{2+} -release (CICR) to the ChR2(H134R)-induced contraction of aortic rings, ryanodine (3 μ M) was added to the PSS solution prior to blue light stimulation to exhaust ryanodine-sensitive Ca^{2+} stores [429]. Figure 5.5A illustrates that this intervention did not significantly affect the arterial response to blue light. Additionally, the role of IP_3 signalling was studied using IP_3 R antagonist 2-APB (60 μ M) (Fig. 5.5A). The role of TRPM4 channels was studied using the blocker clotrimazole (10 μ M) (Fig. 5.5A). Both 2-APB and clotrimazole led to a similar decrease (~ 0.5 - 0.7 reduction) in blue light (< 0.1 mW/mm²) induced contraction of aortic rings isolated from ChR2(H134R)-SM mice. Thus, IP_3 R mediated but not RyR mediated CICR contributed to the blue light induced contraction. However, LVGCC-mediated Ca^{2+} entry is likely to be the prime source of Ca^{2+} leading to arterial contraction in response to blue light (in this defined range) in ChR2(H134R)-SM aortic rings.

CaCC channels, coded by the *Tmem16a* gene, represent a key depolarising mechanism in vascular smooth muscle [218,246,247,430]. CaCCs in SMCs are

typically activated by IP₃-mediated Ca²⁺ release [218,246,247,430]. To investigate whether TMEM16A channels participate in the blue light induced contraction in arteries obtained from Chr2(H134R)-SM mice, the TMEM16A inhibitors MONNA (3 μ M) [240] or Ani9 (1 μ M) [239] were added to the PSS. Pharmacological blockade of TMEM16A channels using these agents significantly reduced the contractile response to blue light stimulation by ~0.6 fold and ~0.4 fold at 0.02 mW/mm² and 0.07 mW/mm², respectively (Fig. 5.5B).

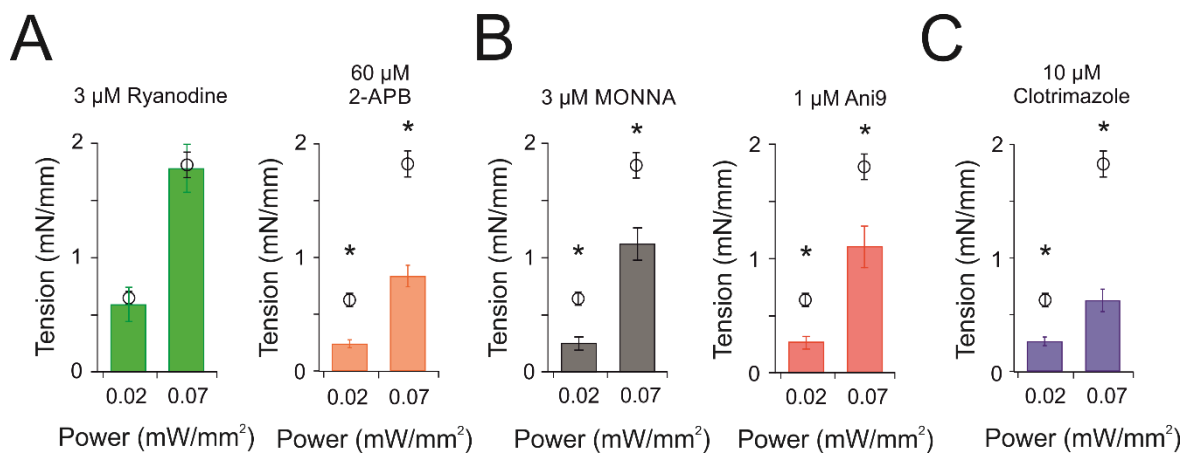


Figure 5.5. Effect of pharmacological ion channel modulation on blue light induced contraction of aortic rings obtained from Chr2(H134R)-SM mice.

A. Mean tension in response to blue light stimulations in PSS supplemented with either 3 μ M ryanodine ($n=6$) or 60 μ M 2-APB ($n=7$), as indicated. Open circles refer to experiments conducted in control PSS solution and are replotted from Fig. 4.8 from Chapter 4. **B.** Mean tension in response to blue light stimulations in PSS supplemented with the TMEM16A blocking agents MONNA (3 μ M) or Ani9 (1 μ M) ($n=7-8$), as indicated. Open circles refer to experiments conducted in control PSS solution and are replotted from Fig. 4.8 from Chapter 4. **C.** Mean tension in response to blue light stimulations in PSS supplemented with 10 μ M of the TRPM channel blocker clotrimazole ($n=7$). Open circles refer to experiments conducted in control PSS solution and are replotted from Fig. 4.8 from Chapter 4

5.2.5 *Light-evoked changes in intracellular pH*

As noted in the Introduction, ChR2(H134R) is a highly H⁺-permeable ion channel; thus, prolonged ChR2(H134R) activation may lead to a net H⁺ flux and changes in pHi. To test this possibility, pHi was measured in isolated aortic SMCs obtained from ChR2(H134R)-SM and control mice before and after 7 min light stimulation of various intensities (Fig. 5.6). The pHi prior to light stimulation was 7.0±0.1 in cells isolated from ChR2(H134R)-SM mice (n=38) and 6.9±0.1 (n=35) in SMCs obtained from control mice (no significant difference). Figure 5.6 shows that at low intensity blue light (<0.1 mW/mm²) no significant change in pHi was observed. At higher intensities, however, light evoked significant alkalinisation of the cytoplasm, of up to ~0.3 pH units (Fig. 5.6B).

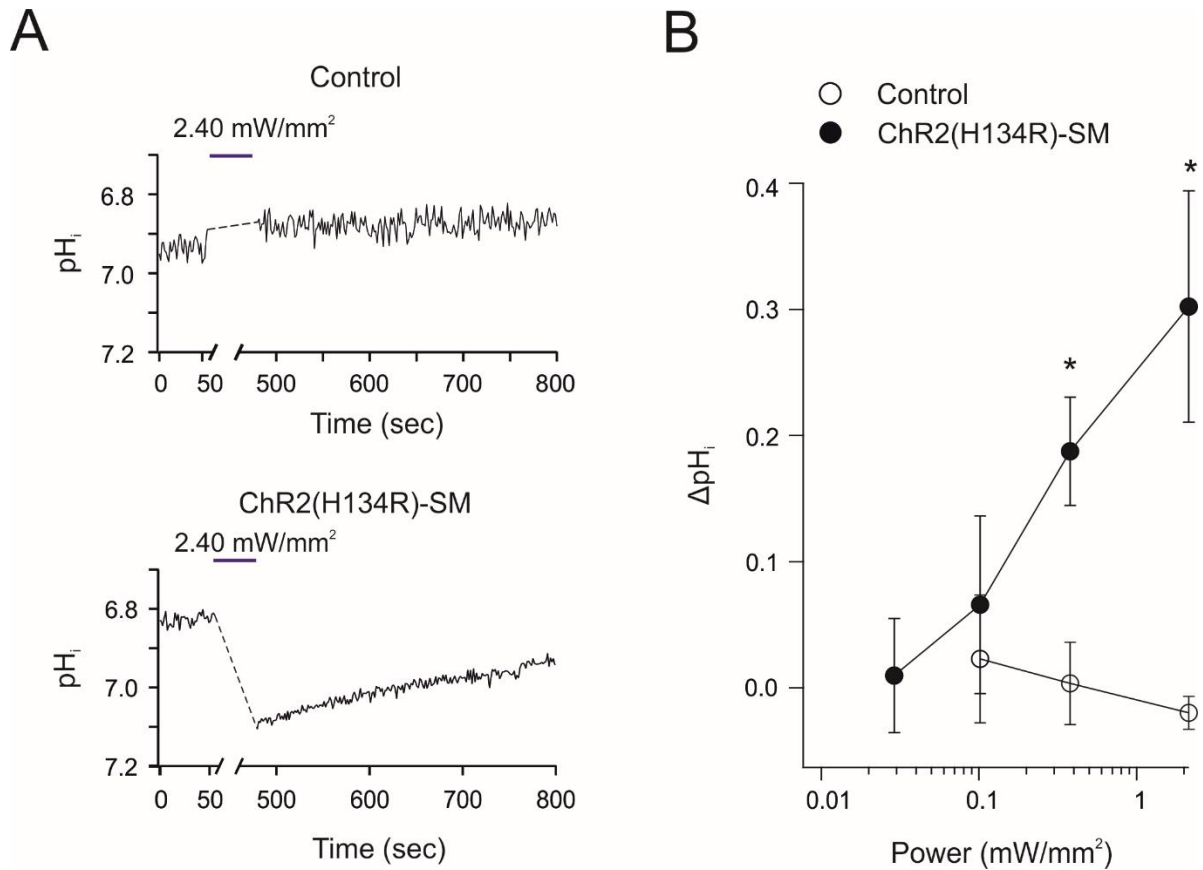


Figure 5.6. Effect of blue light on pH_i of aortic SMCs isolated from control and ChR2(H134R)-SM mice.

A. pH_i measurements in individual aortic SMCs isolated from control (top) or ChR2(H134R)-SM mice (bottom) before and after a 7 min exposure to blue light of different intensities, as indicated. The horizontal blue bars in each panel indicate the 7 min illumination period. **B.** Mean blue light-induced pH_i change versus blue light intensity relationship for SMCs isolated from ChR2(H134R)-SM mice ($n=11-38$) and control mice ($n=12-35$). *, $P<0.05$ (t -test).

5.2.6 Blue light stimulation did not lead to alteration of the pH_e

As established, ChR2(H134R) is proton permeable and activation of the channel leads to an efflux of H^+ from the SMC. To examine if this proton efflux resulted in a change in the pH of the immediate extracellular environment, the pH_e was measured. The change in pH_e in response to blue light (2.4 mW/mm²) was

measured using a pH microelectrode that was placed directly on the surface of aortic rings isolated from control or ChR2(H134R)-SM mice bathed in a low buffer solution ("pH_e solution"; for composition see Chapter 2, Section 2.11.2). The pH_e in the proximity of aortic rings isolated from ChR2(H134R)-SM mice or control mice was not altered in response to blue light stimulation (Fig. 5.7). The blue light induced change in pH_e was -0.02 ± 0.01 pH unit (n=8) change compared to -0.01 ± 0.01 pH (n=5) in aortic rings isolated from ChR2(H134R)-SM or control mice, respectively. The change in pH_e after 7 minutes of blue light exposure was not significantly different in aortic rings isolated from ChR2(H134R)-SM mice compared to the aortic rings isolated from control mice. Thus, it does not appear that the H⁺ current was sufficient to change the pH of the solution surrounding the arterial tissue.

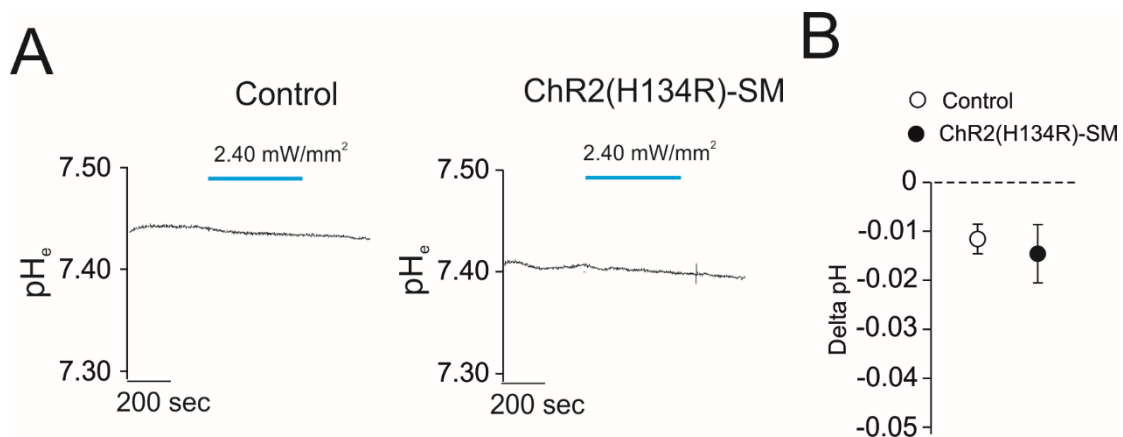


Figure 5.7. Effect of blue light stimulation on pH_e.

A. Representative pH_e recordings of the extracellular solution close to aortic rings isolated from control mice or ChR2(H134R)-SM mice in response to blue light stimulation (2.4 mW/mm², as indicated by the horizontal blue bars). **B.** Mean blue light induced change in pH_e in the solution surrounding aortic rings isolated from control mice (n=5) or ChR2(H134R)-SM mice (n=8).

5.2.7 Comparison of light and agonist-induced contraction

The data shown in Figure 5.1, Figure 5.5 and Figure 5.6 demonstrate the range of blue light intensities within which ChR2(H134R) activation resulted in SMCs depolarisation, LVGCC and TMEM16A activation, and arterial contraction in the absence of pronounced changes in pH_i or Ca^{2+} entry through ChR2(H134R). Under physiological conditions, artery tone can be maintained for extended periods of time in response to continuous stimulus. To investigate whether ChR2(H134R) induced contraction could emulate this, aortic rings obtained from ChR2(H134R)-SM mice were exposed to blue light (0.05 mW/mm^2) for 25 min. This blue light intensity was chosen as it lies within the range of “physiological” light induced contraction mentioned above ($<0.1 \text{ mW/mm}^2$). The blue light induced response was compared to the contraction elicited by the contractile agonists PE ($0.4 \mu\text{M}$) and NA ($10 \mu\text{M}$). The concentration of these agonists were selected as they evoked an average contraction of $1.4 \pm 0.1 \text{ mN/mm}$ (NA, $n=11$) and $1.3 \pm 0.1 \text{ mN/mm}$ (PE, $n=8$), which were comparable to the tension induced by 0.05 mW/mm^2 blue light ($1.2 \pm 0.1 \text{ mN/mm}$ ($n=7$)). Blue light stimulation triggered a contraction that rapidly reached a maximal, stable steady-state value. In contrast, NA produced a transient contraction followed by fluctuating levels of tension (Fig. 5.8A). PE-induced contraction was characterised by a peak followed by a slow drop and then subsequent rise in tension. The nadir to peak ratio was used as a measure of the stability of the tension induced by the various stimuli. This ratio was 0.9 ± 0.1 ($n=7$), 0.3 ± 0.1 ($n=11$) and 0.7 ± 0.1 ($n=8$) for blue light, NA and PE, respectively (Fig. 5.8B). Thus, blue light-stimulation produced a more stable response compared to contractile agonists. This shows that ChR2(H134R) induced contraction can be used to maintain a steady level of contraction for extended periods of time without significant fluctuations in

tone. Thus, blue light induced contraction is suitable for use to control artery tone for pharmacological testing of vasodilators as well as to control artery tone for other experimental purposes for extended periods of time.

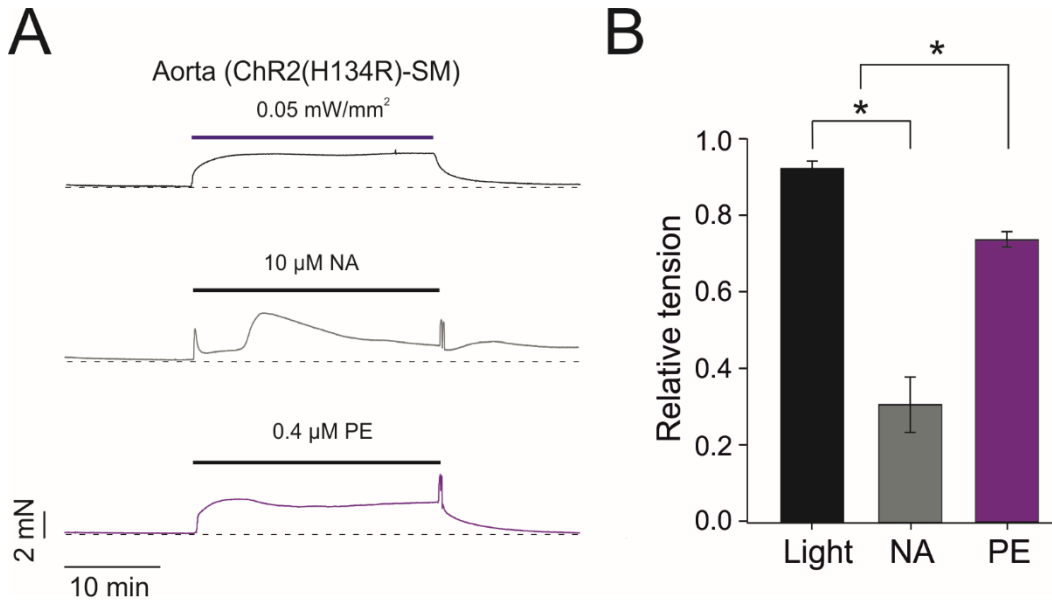


Figure 5.8. Comparison of blue light-induced and agonist-induced contraction of aortic rings obtained from ChR2(H134R)-SM mice.

A. Representative isometric tension recordings obtained from aortic rings isolated from ChR2(H134R)-SM mice. Aortic rings were exposed to blue light, PE or NA for 25 min, as indicated by the horizontal bars. **B.** Mean stability of contraction expressed as the ratio between the maximal and minimal level of contraction elicited by the contractile stimulus ($n=7-11$). *, $P<0.05$ (One-Way ANOVA).

5.2.8 Investigation of the potential functional coupling between SMCs and ECs

One of the key research questions that could be studied through the use of vascular optogenetics is how the electrical coupling between ECs and SMCs affects the function of these cell types and thus how this affects artery tone. It was argued that depolarisation in the SMCs may propagate to ECs through gap junctions [13,24]. An important role of ECs is to release vasoactive factors such as NO (see Chapter

1, section 1.9.2). The production and release of these factors may depend on the EC V_m and $[Ca^{2+}]_c$. Therefore, if the light induced SMC depolarisation spreads to ECs it could affect the NO production, which in turn could alter the blue light induced contraction. Thus, the effect of ChR2(H134R) activation in SMCs in the presence and absence of L-NAME, an eNOS inhibitor, was tested. Isometric tension recordings were conducted using aortic rings obtained from ChR2(H134R)-SM mice that were bathed in PSS supplemented with 100 μ M L-NAME, while exposed to elevated $[KCl]_e$ or 0.07 mW/mm² blue light for 7 min (Fig. 5.9). In the absence of L-NAME, the blue light induced contraction was 2.0 ± 0.2 mN/mm (n=6) while the blue light induced contraction in the presence of L-NAME was 2.3 ± 0.1 mN/mm (n=6) (Fig. 5.9). This was not statistically different. To infer whether the depolarisation spread from the SMCs to the ECs, the light induced contraction was compared to the contraction observed in response to elevated $[KCl]_e$ which would achieve concurrent depolarisation of ECs and SMCs. The contraction elicited by a 10 mM elevation in the $[KCl]_e$ (estimated to give rise to the same magnitude contraction) was 1.4 ± 0.1 mN/mm (n=5) and 2.3 ± 0.1 mN/mm (n=5) in the presence and absence of L-NAME, respectively. This was a statistically significant difference.

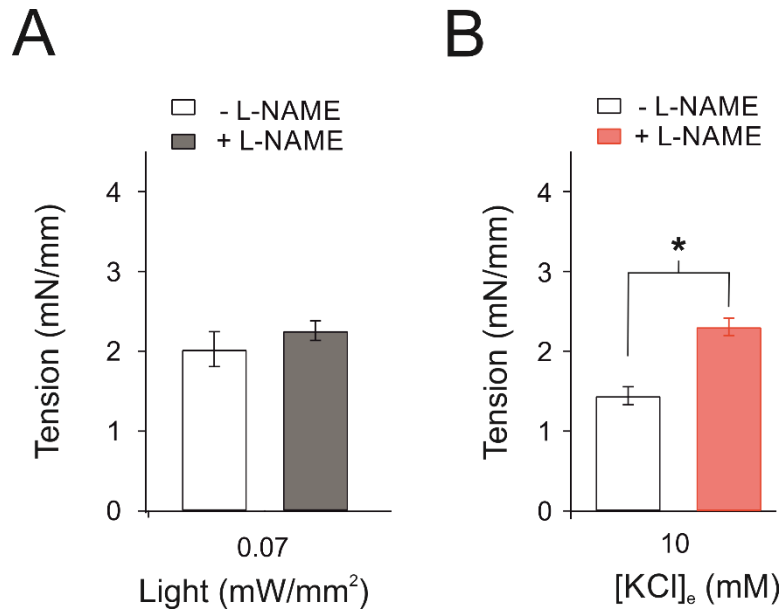


Figure 5.9. Effect of L-NAME on blue light induced or elevated $[KCl]_e$ induced tension of aortic rings obtained from ChR2(H134R)-SM mice.

A. Mean tension normalised for vessel length in response to blue light (0.07 mW/mm²) in the absence or presence of 100 μ M L-NAME (n=6 in each case). **B.** Mean tension normalised for vessel length in response to elevated $[KCl]_e$ (10 mM) in the absence or presence of 100 μ M L-NAME (n=5 in each case).

5.2.9 Towards establishing optogenetics in endothelial cells

To more fully examine the potential for vascular optogenetics in the study of the role of the ECs in relation to the SMCs, a transgenic mouse model selectively expressing ChR2(H134R) in ECs (termed ChR2(H134R)-EC) was generated. Based on the results from the ChR2(H134R)-SM mice, it was argued that the activation of ChR2(H134R) in the ECs would result in V_m depolarisation. The potential for the EC depolarisation to spread to SMCs and thus initiate artery contraction was investigated using this mouse model. The aortic rings isolated from ChR2(H134R)-SM mice saw a 1.5 ± 0.2 mN/mm (n=6) contraction in response to blue light stimulation (0.07 mW/mm²) (Fig. 5.10A). In contrast, aortic rings isolated from

ChR2(H134R)-EC saw a negligible response of -0.01 ± 0.01 mN/mm ($n=4$) (Fig. 5.10A).

To further examine the effect of ChR2(H134R) dependent depolarisation on EC function, the effect of blue light in the presence of $1 \mu\text{M}$ PE was tested. As described in Chapter 4, artery rings isolated from control mice relax in response to blue light stimulus in the presence of PE. The isolated aortic rings obtained from control mice saw a -0.06 ± 0.02 mN/mm ($n=8$) and -0.22 ± 0.06 mN/mm ($n=8$) relaxation in response to 0.03 mW/mm^2 and 0.07 mW/mm^2 , respectively. In comparison, the change in tension observed using aortic rings isolated from ChR2(H134R)-EC mice was negligible in response to 0.03 mW/mm^2 and -0.01 ± 0.03 mN/mm in response to 0.07 mW/mm^2 (Fig. 5.10B).

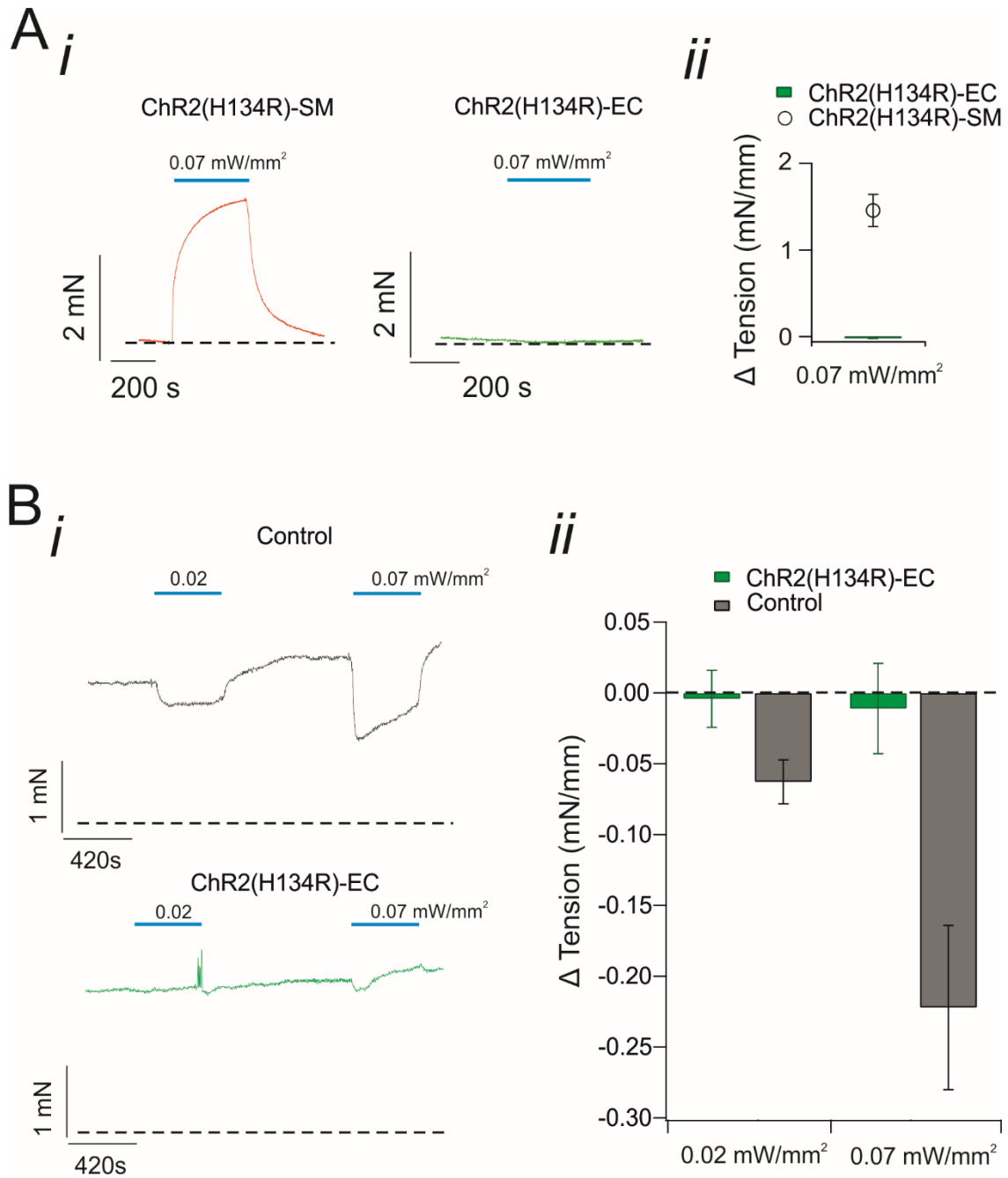


Figure 5.10. Effect of blue light stimulation in the presence or absence of PE in aortic rings isolated from control mice, ChR2(H134R)-SM mice or ChR2(H134R)-EC mice.

A_i. Representative recordings of blue light induced contraction of aortic rings isolated from ChR2(H134R)-SM mice or ChR2(H134R)-EC mice in the absence of PE. **A_{ii}**. Mean change in tension of aortic rings isolated from ChR2(H134R)-SM mice ($n=6$) or ChR2(H134R)-EC mice ($n=4$) in response to blue light stimulation (0.07 mW/mm^2) in the absence of PE. **B_i**. Representative recordings of blue light (0.02 mW/mm^2 or 0.07 mW/mm^2 , as indicated by the horizontal blue bars) induced relaxation from aortic rings isolated from control mice ($n=8$) or ChR2(H134R)-EC mice ($n=4$) in the presence of $1 \mu\text{M}$ PE. **B_{ii}**. Mean change in

tension of aortic rings isolated from control mice or ChR2(H134R)-EC mice in response to blue light stimulation (0.02 mW/mm² or 0.07 mW/mm²) in the presence of 1 μ M PE.

5.3 Discussion

The results shown in this chapter provides a detailed analysis of the mechanism of the blue light sensitivity of isolated vascular SMCs and artery rings obtained from ChR2(H134R)-SM mice. The main findings of this chapter were:

- 1) ChR2(H134R) induced contraction was completely inhibited by nifedipine at blue light at intensities <0.1 mW/mm². At these light intensities, LVGCC activation was mandatory for contraction. In contrast, light stimuli of >0.1 mW/mm² led to contraction even in the presence of LVGCC blockage, indicating that significant Ca²⁺ influx through ChR2(H134R) occurred.
- 2) Within the same range of blue light intensities, TMEM16A channels contributed to the contractile response as evidenced by the significant reduction in the blue light induced contraction with TMEM16A blockers.
- 3) The blue light induced contraction appears to also be dependent on IP₃R signalling and/or TRPM4 activity, as evidenced by the reduced contraction in the presence of 2-APB and clotrimazole, respectively.
- 4) Activation of ChR2(H134R) led to significant alkalinisation at high light intensities. However, at blue light intensities <0.1 mW/mm² the pH_i remained stable, within 0.1 units of resting pH_i.
- 5) There is limited evidence for a spread of blue light induced depolarisation of SMCs to ECs.

- 6) Use of ChR2(H134R)-EC mice indicated that there may be an EC component to the blue light induced relaxation observed in the presence of contractile agonists.

Thus, this study determined the range of light intensities ($< \sim 0.1 \text{ mW/mm}^2$) that enabled modulation of the SMC V_m , and thus artery tone of ChR2(H134R)-SM mice primarily via activation of endogenous LVGCCs while also limiting changes in pH_i .

This experimental analysis reveals the mechanism of ChR2(H134R) induced contraction and the ideal range of light stimulation intensity to be used for experimental purposes; this is instrumental for the complete interpretation of results involving this new technology.

5.3.1 ChR2(H134R)- and agonist-induced contraction of isolated artery rings

Isolated aortic artery rings obtained from ChR2(H134R)-SM mice underwent stable contraction in response to blue light. In contrast, fluctuations of isolated artery tone were observed in response to the agonists PE or NA. This notion may have significant implications for pharmacology and drug discovery. The potency of new vasodilating compounds might be more difficult to assess in vessels pre-constricted with agents that elicit a changing level of vessel tone. Spontaneous variation in tone may complicate the quantification of the effects of vasodilating drugs. The stability of the blue light-induced contraction could therefore aid the assessment of new vasodilating drugs on isolated arteries. Furthermore, optogenetics allows the study of pharmacologically induced vasodilation without concurrent contractile agonist signalling (otherwise required to induce a degree of contraction) that may interfere with the vasodilatory signalling. This optogenetic setting of vascular tone may be

particularly useful for the study of Kv7.x channels which have been suggested as an intermediate step in both contractile and dilatory stimuli [153].

5.3.2 Investigating the ionic mechanisms underlying ChR2(H134R)-induced artery contraction

The study of the ionic mechanism of the light induced contraction was limited to the thoracic aorta. One reason for the centring the study on the aorta was because mouse aorta has been used in many published papers (e.g. [431–437]). This facilitates comparison of the results shown here with previous studies. Another reason why I focussed on the aorta was because of its important role in smoothing the blood pressure (via elastic recoil, as previously mentioned in the Introduction). The aorta is abundantly innervated by the sympathetic nervous system and aortic smooth muscle cell express receptors for factors released by the endothelium (e.g. ET-1) and circulating vasoactive agents (e.g. ATII). Thus, the control of the aortic tone is highly interesting as it could modulate systolic blood pressure and pulse pressure. In this context, it can be envisaged that optogenetics control of aortic tone may be exploited experimentally as a tool to modulate these haemodynamic properties. It would be highly interesting to see whether there are any vessel specific differences, particularly as the expression levels of different ion channels can vary between different artery types (e.g. Kv1.x and RyR subtypes [142,438,439]). Of particular interest would be the resistance arteries as these vessels, as mentioned in the Introduction, play a critical role in regulating the blood pressure and flow in organs. Despite some variation in the expression levels, the key ion channels (e.g. TMEM16A, RyR, IP₃R, TRPM) examined in this study are present in the SMCs of most artery types (e.g. mesenteric, pulmonary, portal vein, aorta and coronary)

[246,439–444]. Thus, it can be presumed that the ionic mechanisms behind ChR2(H134R) induced contraction remains the similar across different artery types.

The experiments involving the use of the nominally Ca^{2+} -free PSS showed that solely blue light induced depolarisation was not sufficient to cause contraction but that extracellular Ca^{2+} needs to enter the cell. However, it is important to note that, as the Ca^{2+} -free PSS solution did not contain EGTA or any other Ca^{2+} chelator, there could indeed be some free Ca^{2+} (10-65 μM) in the solution (Personal communication with Professor Sitsapesan, University of Oxford). Despite this, there is no contraction seen, likely indicating that this concentration of extracellular free Ca^{2+} (which is low compared to the 1.8 mM CaCl_2 included in the standard PSS recipe) did not lead to sufficient Ca^{2+} entry from the extracellular environment for the contractile machinery to be activated. This set of experiments showed that Ca^{2+} influx through LVGCCs, ChR2(H134R) or other channels is required.

LVGCCs are a principal and well established route of Ca^{2+} influx in vascular SMCs [165,421]. As described in the previous chapter, the blue light stimulation resulted in a shift of the V_m into a range where LVGCCs were active. Therefore, ChR2(H134R) primarily served as a switch for LVGCC activation. Application of the LVGCC inhibitor, nifedipine, shifted the tension *versus* light-intensity response curve to the right by about one order of magnitude. At light intensities $<0.1 \text{ mW/mm}^2$, nifedipine completely prevented the blue light induced contraction of aortic rings isolated from ChR2(H134R)-SM mice. However, at higher light intensities a significant Ca^{2+} current may be directly mediated by ChR2(H134R) and thus triggering contraction even in the presence of nifedipine. This theory is supported by the fact that contraction induced by elevated $[\text{KCl}]_e$ was almost completely abolished. This non-physiological Ca^{2+} entry could potentially lead to Ca^{2+} overload,

which could cause functional and histological alterations in the vessel wall [445,446]. The effect of nifedipine was also shown not to be as a result of pharmacological interference with ChR2(H134R) (see Chapter 3 Section 3.2.5). Nifedipine significantly reduces spontaneous TxA2 release from ECs and from platelets [92,447]. As the vessels tested in this study were washed before experiments were conducted, it can be assumed that the platelet derived portion of TxA2 was minimal. A reduction of TxA2 from ECs could potentially further reduce the arterial tone. However, as the concentration to achieve this reduction in TxA2 is higher (~3 μ M) than the nifedipine concentration used in this study it is unlikely that this would have a significant impact [92].

Ryanodine receptor mediated Ca^{2+} -induced Ca^{2+} release (CICR) was not a prime mechanism underlying the blue light-induced artery contraction, as demonstrated by the fact that ryanodine did not alter the extent of blue light induced contraction. This suggests that ChR2(H134R) is not directly juxtaposed to RyR in the SR, as such close proximity between the two channels would likely have given rise to RyR mediated CICR following Ca^{2+} influx through ChR2(H134R). In contrast, the application of the IP_3R antagonist 2-APB reduced the blue light induced contraction significantly. However, this compound, while the most suitable for these experiments done on isolated whole arteries, displays some unspecificity and blocks members of the TRP channel family [448–450]. To try to dissect the cause of the reduction of the contraction, a TRPM8 antagonist (clotrimazole) was used. The reduction in contraction under these conditions was very similar to the reduction seen in the presence of 2-APB. This suggests that the 2-APB might have affected the contraction by inhibiting the non-selective cation channel TRPM8. Other compounds exist that are commonly used to inhibit IP_3R . However, the need for the

agents to act on intact tissue limited the range to 2-APB and Xestospongin C. The efficacy of the frequently used IP₃R blocker Xestospongin C to block IP₃Rs [451,452] has been questioned in a paper dedicated to the study of IP₃R blockers [453] and elsewhere ([62]). It is possible that results previously attributed to IP₃R inhibition are in fact due to SERCA inhibition, which Xestospongin C readily inhibits [454]. However, the promiscuous nature of both these compounds make this analysis difficult and thus this aspect could benefit from further pharmacological studies.

The effects of 2-APB on light-induced contraction may, in part, be due to inhibition of TRP channels, including TRPC channels [449]. It is noteworthy, however, that TRPC channels are especially activated by PIP₂ breakdown, which typically occurs during G_qPCR stimulation [134]. In contrast, in our experiments contraction was caused by Chr2(H134R)-induced membrane depolarisation, during which TRPC channel might be less active (as PIP₂ levels presumably remain unchanged) and thus contribute less to this contraction [134,455].

Block of gap-junction by 2-APB can occur [456]. However, one of the most abundantly expressed connexin in smooth muscle is Cx43 [24] has relatively low sensitivity to 2-APB (IC₅₀ ≈ 52 μM) which would limit the impact of this effect. Furthermore, as the aortic vessel segments are stretched during the isometric vessel tension recordings, the light reaches the tissue uniformly on tissues, so we would expect cells to become activated concurrently and to a comparable degree thus negating any impact the gap junctions may play in the spread of depolarisation between SMCs. The impact of 2-APB on the EC-EC and EC-SMC gap junctions in mice (through Cx40 and Cx37, and Cx43, respectively (reviewed in [24])) could

potentially affect the contractile response. To make more concrete conclusions about the potential effects of the gap junction block the data presented here could be compared to data obtained from experiments using a gap junction specific inhibitor. From this comparison, one could extrapolate to discern the proportion of the 2-APB effect that stemmed from IP₃R antagonism and from gap junction inhibition.

Clotrimazole is a generic TRPM blocker that also acts on IK_{Ca} channels and LVGCCs (in the heart [457]). These side-effects make interpreting the clotrimazole data somewhat more problematic. A possible effect of clotrimazole on IK_{Ca} channels may counteract the inhibition of light-induced contraction. This is because IK_{Ca} channels are especially expressed in endothelial cells, where they contribute to nitric oxide (NO) generation and endothelium-dependent hyperpolarization [168]. However, their inhibition would presumably enhance contraction, while it was in this study found that clotrimazole reduced light-induced contraction.

Furthermore, clotrimazole (at 10 μ M) leads to an estimated ~20% block of cardiac LVGCCs [457]. If this block were to occur in vascular LVGCCs it might, in part, contribute to the inhibitory effects of clotrimazole on light-induced contraction.

The lack of specific TRPM blockers without off-target effects presents a challenge in identifying the cause of the clotrimazole induced reduction in contraction seen in response to blue light stimulation. One potential route could be to use other TRPM blockers that, while they might have other off-target effects, do not act on IK_{Ca} or LVGCCs and compare the results to the clotrimazole data. One potential alternative drug is 9-Phenanthrol [458]. However, as this drug has been shown to block CaCCs

at similar concentrations required for TRPM block [459] it may require comparison with the data collected from the Ani9 and MONNA experiments.

The TMEM16A channel blockers Ani9 and MONNA reduced the blue light induced contraction of isolated aortic rings. This may suggest that CaCC currents participate in the blue light induced contraction by depolarising the cell following activation by Ca^{2+} entering the cell through LVGCCs or ChR2(H134R). TMEM16A channels act as depolarising channels in SMCs due to the fact that the intracellular Cl^- concentration ($[\text{Cl}^-]_i$) in SMCs is maintained high by the presence of active transport mechanisms such as $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ co-transporter (NKCC1) and $\text{Cl}^-/\text{HCO}_3^-$ exchanger (AE2) [123,219]. This elevates equilibrium potential for Cl^- above the resting potential of the cell [218,246,247,430]. Thus, TMEM16A opening may contribute to the light-induced depolarisation of SMCs and arterial contraction. However, in SMCs the E_{Cl} is ~ -25 mV; therefore, at maximal light intensity the V_m reached would be more depolarised than the E_{Cl} and would thus result in an influx of Cl^- which would serve as a brake for further depolarisation.

5.3.3 *Light-driven intracellular alkalinisation*

ChR2(H134R) is highly H^+ permeable [295,304,306,311,366]. We demonstrated that prolonged ChR2(H134R) activation in response to high intensity (>0.1 mW/mm²) blue light stimulations led to intracellular alkalisation. These changes in pH_i are consistent with the fact that the average equilibrium potential for H^+ (E_{H}) in the aortic SMCs was approximately -28 mV under our experimental conditions. Thus, when V_m rises to values above -28 mV, as occurs in response to blue light intensities >0.1 mW/mm², outward H^+ current through ChR2(H134R) is expected to promote an elevation in pH_i which was indeed observed. We argue that a small

depolarisation of the V_m below ~ -28 mV, associated with blue light intensity lower than ~ 0.1 mW/mm², did not lead to significant acidification of pH_i as ChR2(H134R) were only partially activated (i.e. had a low open probability) at these blue light intensities. Furthermore, the abundant expression of Na⁺/H⁺ exchanger in SMCs [460] is likely to correct for H⁺ influx through ChR2(H134R) channels.

The effect of a change in pH_i by >0.1 pH units can significantly alter the sensitivity of SMCs to humoral factors [367,368,461,462] as well as affect SMC migration [462] and gene transcription [367,463]. Thus, prolonged activation of ChR2(H134R) by high intensity blue light *in vivo* may lead to a pH-dependent alteration in arterial histology and function including the extent of contraction through modulation of LVGCCs, capacitative Ca²⁺ entry, and SERCA activity [367,461,464]. Thus, H⁺ flux may produce more complex effects on vascular biology than solely its effects on the V_m .

In addition to changes in pH_i we explored the possibility that the H⁺ efflux could alter the pH of the surrounding tissue. The pH_e is known to alter EC function and stimulate NO production [465]. However, as demonstrated by the pH_e recordings, no change was observed after extended exposure to high intensity blue light.

5.3.4 Electrical coupling between SMCs and ECs

The ECs and SMCs in the artery wall are functionally coupled through locally released factors as well as electrically [13]. The difference between the global depolarisation by elevated $[KCl]_e$ and the specific depolarisation of the SMCs using optogenetics suggests that the depolarisation does not spread from SMCs to ECs. However, V_m depolarisation using ChR2(H134R) in ECs could be expected to have a smaller effect on the $[Ca^{2+}]_c$ than in SMCs due to the absence of LVGCCs [13].

Therefore, while EC function is highly dependent on $[Ca^{2+}]_c$ it is comparatively uncoupled from the V_m . The increase in $[Ca^{2+}]_c$ required for the activation of eNOS and NO production is instead much more extensively mediated by CICR, GPCR signalling and non-selective channels [13,28].

The selective expression of ChR2(H134R) in EC did not lead to contraction of isolated artery rings. This suggests that the selective depolarisation of ECs did not spread to SMCs (at least not to the extent as to stimulate the opening of LVGCCs). In the presence of contractile agonists such as PE, native arteries relax in response to blue light stimulation [358,359,361]. This is known to involve the activation of melanopsin in the SMCs [361]. However, the lack of the blue light induced relaxation in the aortic rings isolated from ChR2(H134R)-EC mice may indicate that there is an endothelial component to this relaxation which is inhibited by EC V_m depolarisation.

5.1.1 Implications for vascular physiology

Vascular optogenetics has many potential applications including serving as an experimental tool to control blood perfusion to specific organs or tissues to manipulate the metabolic rate temporospatially by reducing O_2 and nutrients supplied [363]. Optogenetically induced organ ischemia could potentially be experimentally reproduced by inducing vessel constriction *in vivo* in experimental animals, potentially via the use of remotely controlled wireless micro LEDs implanted subcutaneously [466]. Utilising these wireless micro LEDs has already been used for deep brain stimulation to optogenetically induce muscle twitching [467]. This may have important implications for biomedical research by aiding the identification of new drugs or treatments that could counter the ischaemia.

Restricting blood flow could be particularly useful in the study of disease states, such as diabetic retinopathy, glaucoma and retinal vessel occlusion, in which the retinal blood flow is impaired leading to loss of vision [468–470]. The stable contraction obtained in isolated arteries may also facilitate drug testing *ex vivo*. At a single cell level, the possibility of controlling V_m optically could enable high-throughput screening of drugs at various V_m , including depolarised V_m typically associated with diseases of altered vessel tone [471,472]. Our study demonstrates that these applications are especially viable within a well-defined range of blue light of low intensity within which ChR2(H134R) activation serves as a mere switch for activation of endogenous membrane transport mechanisms. It can be envisaged that viral-mediated ChR2(H134R) expression could be obtained in cultured human cells (or stem cell derived SMCs) to be used in drug testing. This may help reducing, and to an extent replacing, the use of animals during drug screening studies.

Vascular optogenetics has the potential to further our understanding of the physiological role of electrical coupling between different cell types (such as SMCs and ECs) within the arterial wall. Investigating the electrical coupling between ECs and SMCs in intact vessels has previously presented significant technical challenges. For instance, direct injection of current in ECs using sharp microelectrodes may result in non-uniform control of membrane potential, whilst the limited current-passing ability of the microelectrodes restricts the range of potentials over which the membrane can be clamped. Furthermore, electrical stimulation may also trigger irreversible Faradaic reactions, potentially leading to the formation of gases such as H_2 , O_2 or Cl_2 , and ROS, which are likely to harm the cell or interfere with signalling pathways (as described in the Introduction) [334]. Optogenetics may

enable a non-contact stimulation of specific cell types in arterial wall for prolonged periods of time and in the absence of the above-mentioned limitations.

This optogenetic mouse model could additionally be used for the study of different, non-vascular, SMC types. One potential application of controlling SMCs is in treating incontinence as the application of light to the inner rectal sphincter could result in its closing. This could be particularly useful for patients with spinal cord injury that may result in loss of control of the lower body.

5.3.5 Conclusion

The results shown in this chapter shows that vascular optogenetics based on ChR2(H134R) has some limitations. These could potentially be bypassed by using a recently generated Cl⁻ selective and H⁺ impermeable ChR2 mutant named iChloC [315]. Due to the high intracellular Cl⁻ concentration in SMCs [123,218,430], activation of a light-activated Cl⁻ channel is expected to evoke V_m depolarisation, activation of LVGCCs and hence smooth muscle contraction. Thus, we envisage that iChloC could serve as a depolarising mechanism without the changes in pH_i associated with the H⁺ permeability of ChR2(H134R) or the potential Ca²⁺ overload due to ChR2(H134R)-mediated Ca²⁺ current. The use of iChloC may enable extension of vascular optogenetics to a much broader range of light stimulations and, consequently, enable a broader extent of artery tone regulation. There is no doubt that optogenetics has the potential to trigger new investigations in vascular research. It can be envisaged that opsins that cause V_m hyperpolarisation might lead to vessel relaxation. These opsins might have the potential to be utilised in gene therapy in human subjects. This might have important therapeutic implications

for the treatment of widespread vascular diseases associated with altered vessel tone.

Chapter 6

Characterisation of a Cl⁻-selective ChR2 variant

6.1 **Introduction**

As demonstrated in the previous chapters, ChR2(H134R) expressed in SMCs elicit prominent depolarising currents that lead to arterial muscle contraction. It was also shown that, at high light intensities, ChR2(H134R) activation provoked Ca²⁺ entry through ChR2(H134R) and dramatic pHi changes.

In 2014, Wietek *et al.* identified a single point mutation (substitution of a glutamate at position 90 with an arginine) in ChR2 that render the channel selective to anions. In 2015, the same research group identified additional residues (E83Q and E101S) that improved the Cl⁻ selectivity of the channel. Additional, already defined mutations (D156N and T159C) were also included to increase the photocurrent amplitude. This variant, harbouring five mutations, was termed iChloC. In these initial papers, however, the exact light dependence of these mutant channels and their selectivity to proton was not systematically assessed. The work presented in this chapter represent the first extensive biophysical characterisation of iChloC. Specifically, the relationship between light intensity and photocurrent amplitude was explored, and the impact of this current on the V_m of the transfected HEK293T cells was established. Finally, the H⁺ and anion selectivity of this channel was accurately assessed.

6.2 **Results**

6.2.1 *iChloC mediated photocurrents exhibit a delayed onset and recovery from blue light stimulation*

For iChloC to be a suitable candidate for use in vascular optogenetics it needs to be able to mediate depolarising currents that vary in amplitude with the stimulatory light intensity. Photocurrents in HEK-293T cells expressing iChloC were measured using the whole-cell patch-clamp technique. To allow comparison between different size cells, the whole-cell current was normalised for the cell capacitance to obtain the current density [382,406]. iChloC currents were elicited by blue light stimuli of varying intensity (0.01 mW/mm² to 12.1 mW/mm²) at a V_m of -80 mV. Note that in these experiments, the stimulation period and the recovery period were increased to 10 and 40 s for iChloC (compared to the 2 and 4 s used in the experiments involving ChR2(H134R)). This change was done because my preliminary experiments (not shown) and previous published work [292] showed that iChloC appeared to present slower activation and deactivation rates. The current amplitude increased in a blue light intensity dependent manner. The current observed at the lowest light intensity tested was -8.1 ± 1.9 pA/pF ($n=8$) (i.e. ~45% of the current seen at the maximal light intensity tested). In comparison, the ChR2(H134R) mediated current was very low at the lowest light intensity (see Chapter 3, Section 3.2.1). The fit of the iChloC mediated current *versus* blue light intensity relationship with Eq. 2.3 yielded an EL_{50} of 0.02 ± 0.01 mW/mm², a slope factor (h) of 0.51 ± 0.06 and a maximal asymptotic current (I_{max}) of -20 ± 2.1 pA/pF.

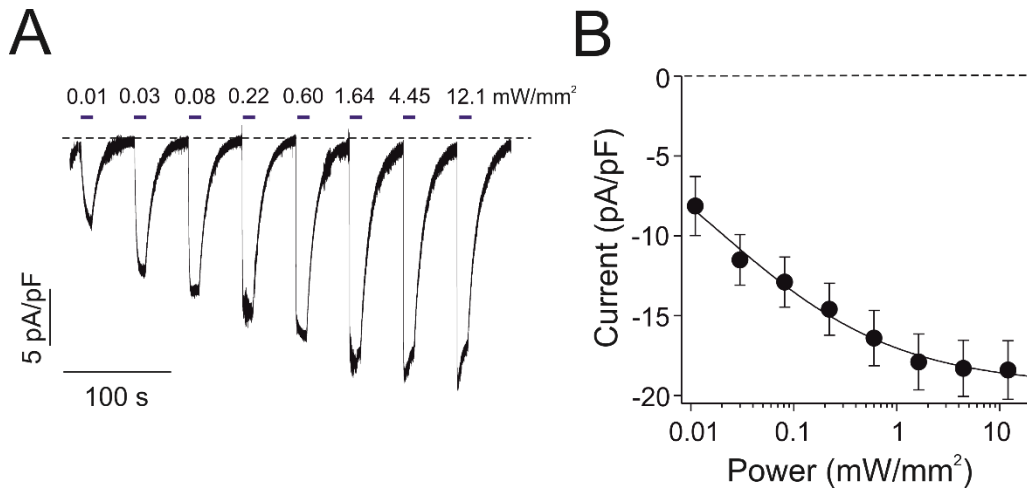


Figure 6.1. Electrophysiological analysis of *iChloC* currents in the HEK-293T expression system.

A. Representative whole-cell current density from HEK-293T cells expressing *iChloC*, in response to blue light stimulation (as indicated by the horizontal blue bars). The V_{hold} was -80 mV. The dashed horizontal line represents the zero-current level (dark current). **B.** Mean whole-cell current density versus blue light intensity relationship measured at the end of each blue light exposure for HEK-293T cells expressing *iChloC*. Smooth line through points represent the best fit of the data with the Hill equation (Eq. 2.3). The number of experiments was 8.

6.2.2 Extended exposure to blue light

As showed in Chapter 4 and Chapter 5, and consistent with published work [143,362], vascular SMCs contract slowly and vessel tone can require minutes to fully develop. Thus, for *iChloC* to be suitable for vascular optogenetics, it needs to be able to generate a stable current for several minutes. *iChloC* expressing HEK-293T cells were exposed to blue light of intensity 0.08 mW/mm² for 7 min at a constant V_m of -80 mV and the current recorded during whole-cell patch-clamp. This duration was chosen because, as shown in Chapter 4, 7 minutes is the time required for an isolated aortic ring to reach a stable contraction in response blue light or to elevated (50 mM) KCl. Figure 6.2 shows that the photocurrent amplitude

remained stable throughout the exposure period. The mean change in photocurrent current density between the first and seventh minute of the stimulation was 1.6 ± 0.7 pA/pF.

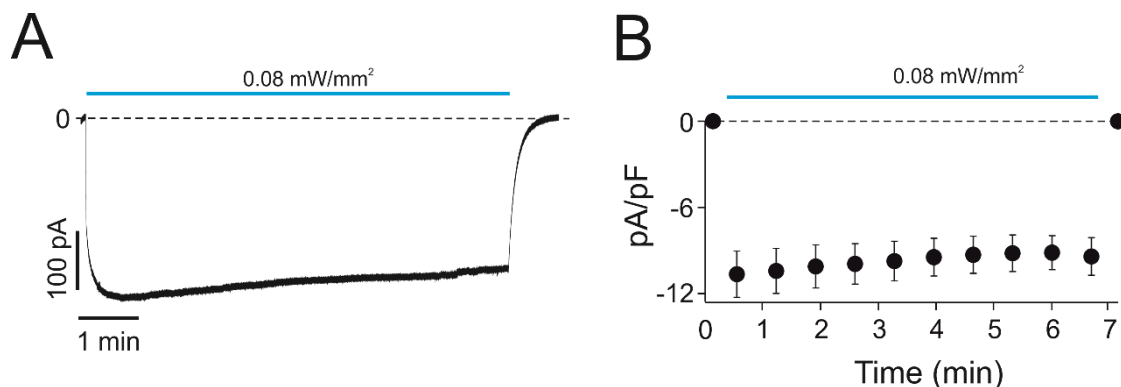


Figure 6.2. Effect of extended blue light exposure on *iChloC* current amplitude.

A. Representative whole-cell currents in HEK-293T cells expressing *iChloC* evoked by extended exposure to blue light (represented by the horizontal blue bar). **B.** Mean current density of *iChloC* throughout the extended exposure to blue light (represented by the horizontal blue bar). The number of experiments was 5.

6.2.3 Light evoked changes in V_m of *iChloC* expressing HEK-293T cells

To assess the capacity of *iChloC* to alter the V_m of HEK-293T cells was assessed in current-clamp experiments. Experiments were conducted in solutions that mimicked the physiological $[Cl^-]_e$ and $[Cl^-]_i$ in SMCs (resulting in an E_{Cl} of -20 mV). Under these conditions, activation of *iChloC* led to V_m depolarisation (Fig. 6.3). Blue light stimulus (10 s duration, elicited every 40 s) of increasing intensity (from 0.01 mW/mm² to 12.1 mW/mm²) led to increasing levels of V_m depolarisation (Fig. 6.3). In high $[Cl^-]_i$ conditions, the mean resting V_m was -25 ± 2.9 mV and a mean maximal depolarisation of 10 ± 0.6 mV ($n=4$) was observed in response to the maximal light intensity tested (12.1 mW/mm²). Cessation of light stimulation resulted in return to resting V_m (Fig. 6.3A). The fit of the V_m versus light intensity relationship with Eq.

2.4 yielded an EL_{50} of 0.02 ± 0.01 mW/mm² (n=4), a slope factor (ν) of 1.3 ± 0.7 (n=4) and the maximal asymptotic V_m (V_{max}) of -9.5 ± 2.8 mV (n=4).

To verify that depolarisation mediated by iChloC was due to Cl⁻ currents, whole-cell current-clamp experiments were also conducted in low [Cl⁻]_i conditions (resulting in E_{Cl} of -69 mV). The prediction was that, under these conditions, opening of iChloC would cause V_m hyperpolarisation. Exposure to blue light resulted in hyperpolarisation of iChloC expressing HEK-293T cells. A mean maximal hyperpolarisation of about ~15 mV was observed (from a mean resting V_m of -28 ± 2.8 mV) in response to the maximal light intensity tested (12.1 mW/mm²). Removal of the blue light stimulus resulted in a return to the resting V_m . The fit of the mean data using Eq. 2.4 yielded an EL_{50} of 0.04 ± 0.02 mW/mm² (n=6), a slope factor (ν) of 0.5 ± 0.1 (n=6) and the maximal asymptotic V_m (V_{max}) of 40 ± 3.7 mV (n=6). Thus iChloC cause V_m changes by mediating Cl⁻ currents. There was no light induced change in V_m in non-transfected cells (2.0 ± 0.4 mV at 12.1 mW/mm²; n=5) (not shown).

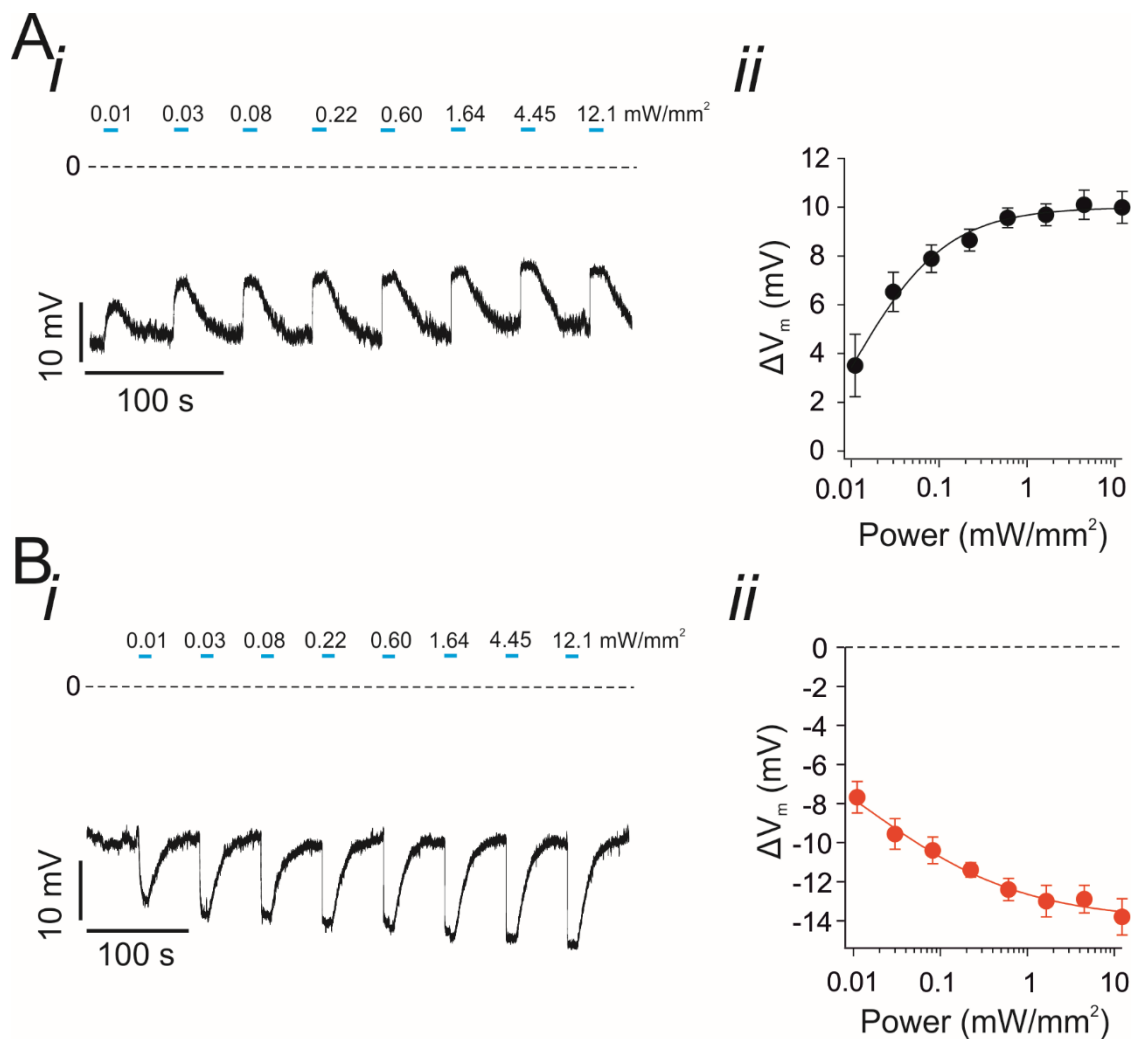


Figure 6.3. Blue light dependent changes in V_m .

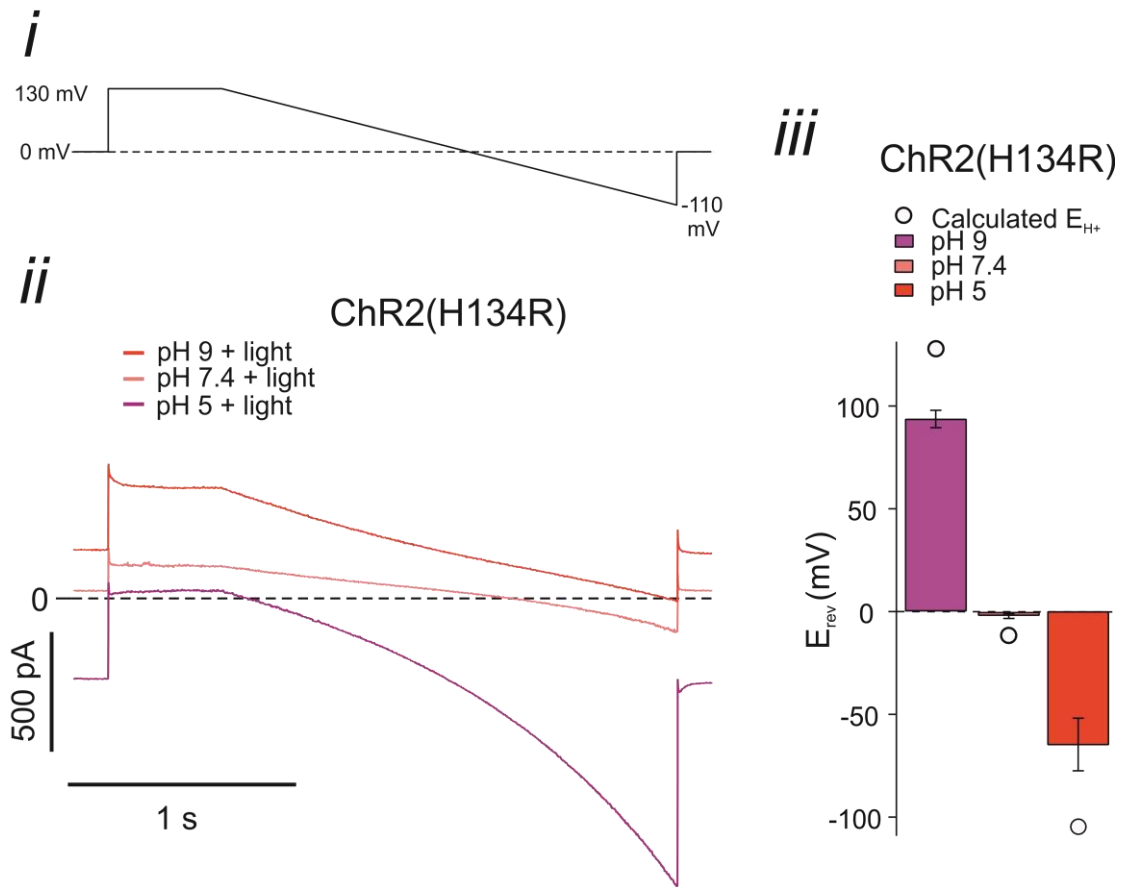
Ai. Representative trace of blue light induced V_m depolarisation of a iChloC expressing HEK-293T cell in high $[Cl^-]_i$ conditions (66 mM). The horizontal blue bars indicate the blue light application periods. The intensity of the blue light stimulations is noted above these blue bars. **Aii.** Mean blue light induced change in V_m of iChloC expressing HEK-293T cells in high $[Cl^-]_i$ (66 mM) conditions ($n=4$). The curve through filled circles represents the best fit of the data with Eq. 2.4. **Bi.** Representative trace of blue light induced V_m hyperpolarisation of a iChloC expressing HEK-293T cell in low $[Cl^-]_i$ conditions (13 mM). The horizontal blue bars indicate the blue light application periods. The intensity of the blue light stimulations is noted above these blue bars. **Bii.** Mean blue light induced change in V_m of iChloC expressing HEK-293T cells ($n=7$) in low $[Cl^-]_i$ (13 mM). The curve through filled circles represents the best fit of the data with Eq. 2.4.

6.2.4 Proton selectivity of ChR2(H134R) and iChloC

As mentioned in Chapter 5, one of the main drawbacks of ChR2(H134R) in vascular optogenetics is the flux of H⁺ ions that occur when the channel is activated by high power blue light. The H⁺ permeability of iChloC in comparison to that of ChR2(H134R) has never been tested. Thus, a side-by-side comparison of these properties was performed.

HEK-293T cells expressing either ChR2(H134R) or iChloC were exposed to blue light (0.6 mW/mm²) while being perfused with extracellular solutions with different pH (5, 7.4 or 9) (for composition see Chapter 2, Section 2.10.2). The E_{rev} of the ChR2(H134R) current was 94±5.5 mV (n=12) in pH 5, -3.2±1.5 mV (n=13) in pH 7.4, and -69±12 mV (n=7) in pH 9 (Fig. 6.4A). In contrast, there was no significant change in the reversal potential in iChloC expressing cells; the E_{rev} was -13±2.1 mV, 13±2.0 and 11±1.7 mV for pH 5, pH 7.4 and pH 9, respectively (n=7 in all cases) (Fig. 6.4B).

A



B

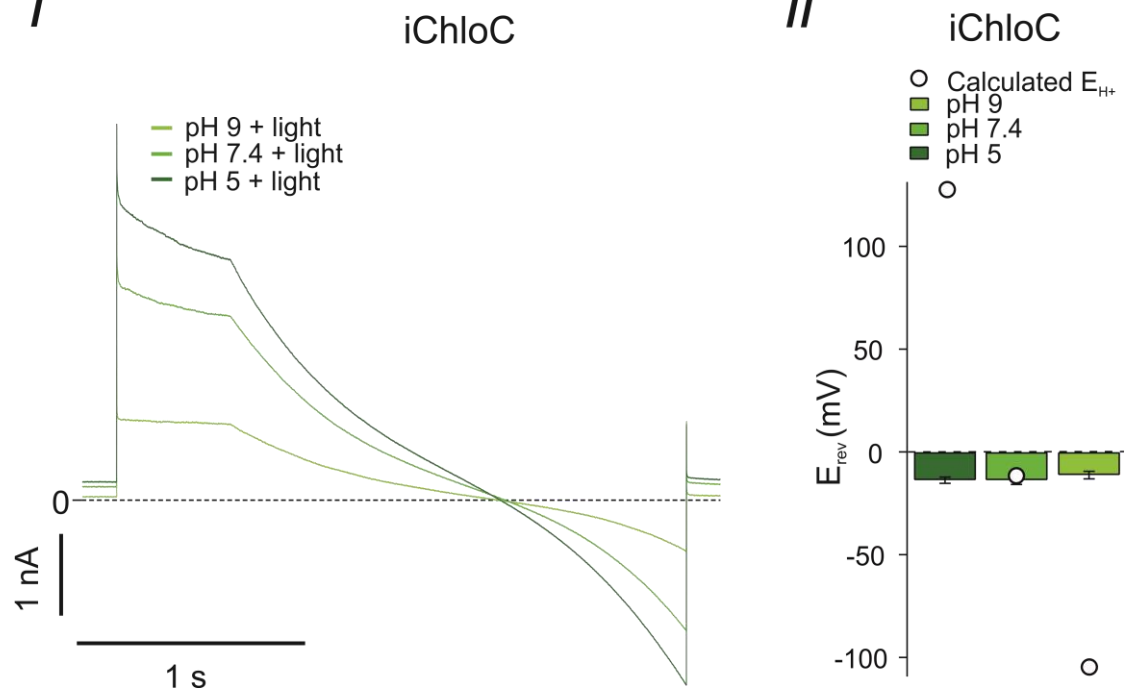


Figure 6.4. H^+ selectivity of ChR2(H134R) and iChloC.

Ai. Voltage ramp protocol used. **Aii.** Representative currents from ChR2(H134R) expressing HEK-293T cells, in response to the ramp protocol shown in **i**, under varying conditions. The cells were exposed to pH 5, pH 7.4 and pH 9 while illuminated with blue light (0.60 mW/mm²). All solutions contained 10 μ M DIDS to block native acid activated currents. **Aiii.** Mean E_{rev} of the current elicited by the voltage ramp protocol in the different pH solutions. The calculated E_H in the different conditions is indicated by the open circles. **Bi.** Representative currents from iChloC expressing HEK-293T cells, in response to the ramp protocol shown in **i**, under varying conditions. The cells were exposed to pH 5, pH 7.4 and pH 9 (while illuminated with blue light (0.60 mW/mm²)). All solutions contained 10 μ M DIDS to block native acid activated currents. **Bii.** Mean E_{rev} of the current elicited by the voltage ramp protocol in the different pH solutions. The calculated E_H in the different conditions is indicated by the open circles.

6.2.5 Anion selectivity of ChR2(H134R) and iChloC

The selectivity of ChR2(H134R) and iChloC for Cl⁻ ions was assessed by measuring the shift in E_{rev} of the current elicited by an IV ramp in HEK-293T cells expressing either iChloC and ChR2(H134R). These cells were exposed to blue light stimulation (0.60 mW/mm²) while being perfused with extracellular solutions where the main anion was either Cl⁻ or gluconate (composition of the solution is described in Chapter 2, Section 2.11.4). In the solution in which the main anion was Cl⁻ the E_{rev} of the photocurrent was -9.3 ± 1.8 mV (n=7) and 1.1 ± 1.8 mV (n=8) for iChloC and ChR2(H134R), respectively. These values differed from the calculated E_{Cl} (-20 mV, indicated by the open circles in Figure 6.1), but the E_{rev} for iChloC expressing cells was closer to the E_{Cl} than cells expressing ChR2(H134R).

When perfused with an extracellular solution containing gluconate as the primary anion, the E_{rev} of the currents observed was 1.8 ± 1.6 mV (n=8) for ChR2(H134R) expressing cells and 30 ± 1.3 mV (n=4) for iChloC expressing cells. The E_{Cl} , as calculated by the Nernst equation, was 46 mV under these conditions. Thus, Cl⁻

currents (mediated by iChloC or native Cl⁻ channels) would experience a shift in the E_{rev} under these conditions.

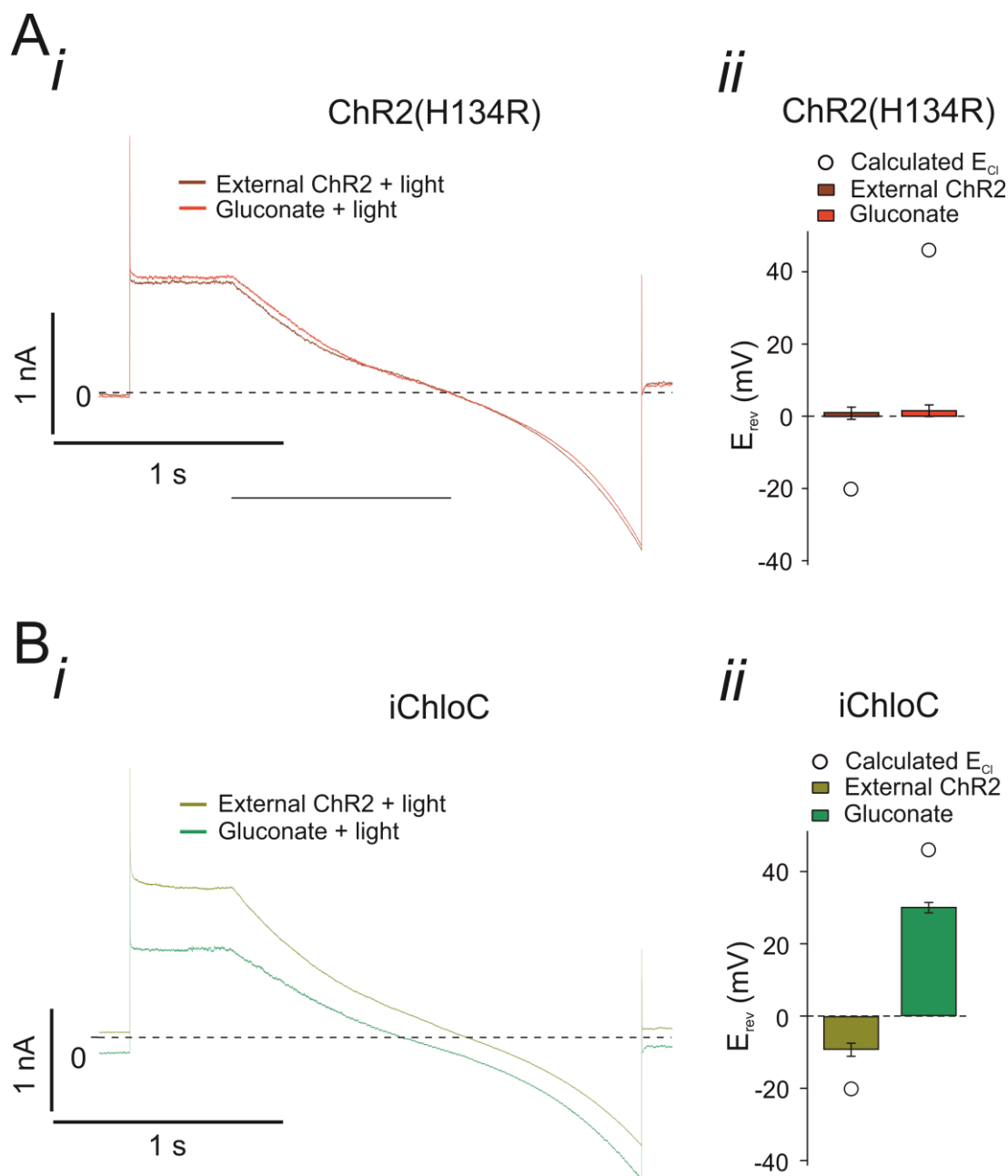


Figure 6.5. Anion selectivity of ChR2(H134R) and iChloC.

Ai. Representative currents from ChR2(H134R) expressing HEK-293T cells in response to a ramp protocol from 130 mV to -110 mV (see Fig. 6.4A) under varying conditions. The cell was exposed to solutions containing either Cl⁻, or gluconate as the main anion while illuminated with blue light (0.60 mW/mm²). **Aii.** Mean E_{rev} of the current elicited by the voltage ramp protocol in the different pH solutions. The calculated E_{Cl} in the different

conditions is indicated by the open circles. **Bi.** Representative currents from iChloC expressing HEK-293T cells in response to a ramp protocol from 130 mV to -110 mV under varying conditions. The cell was exposed to solutions containing either Cl⁻ or gluconate as the main anion while illuminated with blue light (0.60 mW/mm²). **Bii.** Mean E_{rev} of the current elicited by the voltage ramp protocol in the different pH solutions. The calculated E_{Cl} in the different conditions is indicated by the open circles. The liquid junction potentials were corrected for offline as described in the Methods.

6.3 Discussion

The main findings of this chapter were:

- 1) iChloC has enhanced sensitivity to blue light;
- 2) iChloC mediates a long-lasting, non-inactivating Cl⁻ current;
- 3) Opening of iChloC in HEK-293T cells cause a shift of the V_m in the depolarising or hyperpolarising direction, depending on the intracellular Cl⁻ concentration;
- 4) iChloC is impermeable to H⁺ suggesting that opening of iChloC in SMC would not give rise to changes in pH_i , such as those observed when ChR2(H134R) (which is H⁺ permeable) was activated.

Here, I argue that a Cl⁻ selective, but H⁺ and Ca²⁺ impermeable, ChR2 variant could provide SMC depolarisation and artery contraction without an alteration in Ca²⁺ and H⁺ handling, associated with the ChR2(H134R) variant.

6.3.1 *Light causes depolarising Cl⁻ currents in iChloC expressing cells that remain stable for extended periods of time*

Blue light stimulation of cells expressing iChloC led to blue light mediated depolarising currents. iChloC currents activated and deactivated more slowly compared to ChR2(H134R), while being much more sensitive to blue light than ChR2(H134R). The increased sensitivity to blue light stimulation could be advantageous in vascular optogenetics, as it could enable the activation of iChloC in cells that are deep in the vessel wall. The sensitivity of the iChloC could potentially be further increased through the incorporation of mutations that shift the stimulatory light sensitivity towards the far red end of the visible light spectrum. One such mutation, discussed in the introduction, is L171I, which reduces the desensitisation of the channel at 610 and 630 nm and extends the life time of the transient intermediate states of a Channelrhodopsin-1 variant, thus allowing it to be reliably opened by red light [318]. The equivalent residue in ChR2 (upon which iChloC is based) is already an isoleucine residue which may mean that sensitisation to red wavelengths through mutations at this site is not possible [348]. The combination of these variants could potentially result in a new channel that is highly suited to controlling blood vessel tone by being red light activated and Cl⁻ selective, allowing deep stimulation of more “physiological” depolarising Cl⁻ currents (providing a facsimile CaCC current). Rather than red-shifting iChloC one could use another optogenetic tool based on a newly discovered, natively Cl⁻-selective, opsin (PsChR1) which has a shifted absorption spectrum allowing stimulation by green wavelengths of light [473]. This newly discovered channel also mediates a greater amplitude photocurrent compared to iChloC, which may make it more suitable for viral delivery where expression levels can be low (and deteriorate over time [343]).

Lower light intensities than those shown in Figure 6.1 were not attainable with our LED source. To reduce the light intensity further, to achieve a lower degree of light activation, one could potentially incorporate a semi-opaque filter in the light path. If it posed a significant problem, the sensitivity of the channel to blue light could be reduced through the reversal of the T159C mutation (thus reducing the stabilisation of the retinal, leading to reduced sensitivity to light [295]).

iChloC is Cl⁻ selective. Consistent with this notion, when whole-cell current-clamp experiments were conducted in the presence of high Cl⁻ in the pipette, activation of iChloC mediated currents resulted in a ~10 mV depolarisation (i.e. a shift toward the E_{Cl} of ~20 mV that is predicted by the solutions used). It can be anticipated that in SMC, a shift of this magnitude would be sufficient to activate LVGCCs in SMCs [165] by shifting the V_m from ~-45 to ~-35 mV and into the LVGCC window current range (see Chapter 4, section 4.2.2). When experiments were carried out with a pipette solution containing low Cl⁻ (to mimic the intracellular composition of, for example, a skeletal muscle fibre [110]) iChloC activation led to hyperpolarisation. The inhibition of neuronal firing was in fact the reason for its development [292,315].

As discussed in Chapter 1 and Chapter 3, it is desirable for an opsin used in vascular optogenetics to mediate a stable current that can be maintained for several minutes. As seen in this chapter, iChloC mediated stable currents with only a small decrease over time (~10%). Though small, it may be worth keeping this decrease in current in mind if utilising this opsin for extended stimulation protocols as the reduction in depolarising currents over time could result in a decreased arterial tone. This could potentially impact the accuracy of experiments carried out on a longer time scale, for example in the study of vasodilators or when inducing ischaemia (where a

reduced current might lead to partial rather than full occlusion of the artery). The opening and closing kinetics of iChloC are significantly slower than many of the opsins commonly used [293,294,297,304]. This limits its use in excitable cells such as neurones or cardiac myocytes, but it not a significant problem in most populations of SMC as these cells contract more slowly than the time course of iChloC activation. In populations of SMCs in which action potentials have been observed (e.g. in the portal vein [409]) these slower kinetics may make iChloC unsuitable.

6.3.2 Proton selectivity of iChloC and ChR2(H134R)

The experiments described in section 6.2.4 show that altering the pHe does not significantly affect the E_{rev} of iChloC mediated currents. In contrast, the E_{rev} of the ChR2(H134R) current was significantly affected by the pHe. These results indicate that ChR2(H134R) allows a significant H⁺ current, whereas iChloC does not. These results support the hypothesis that iChloC could be used in a vascular setting without causing a change in the pHi. Changes in pHi has many implications in many cell types (in for example neurones and pancreatic β -cells [324,474,475]), thus altering the pHi via optogenetic activation could lead to unwanted changes to the normal cellular function (leading to e.g. excitotoxicity, altered Ca²⁺ handling or altered insulin release [324,474,475]). Thus, the absence of a H⁺ component in the iChloC mediated current is a significant advantage over ChR2(H134R) and will be of interest for researchers in other fields who may want to eliminate unwanted changes in pHi.

The shift in E_{rev} of the ChR2(H134R) mediated current in response to altered pHe was significant. However, the E_{rev} did not reach the E_H calculated using the Nernst equation. This could be due to a number of reasons. One of these would be due to

a leak current due to a poor seal during the whole cell experiment. However, this is not very likely as only cells that maintained a seal resistance of $>1\text{ G}\Omega$ throughout the experiment were included in the analysis. Another potential cause is a non-H⁺ component of the current by one of the other permeable charge carriers included in the solution (Cs⁺, Ba²⁺, Mg²⁺). Though the concentration of these ions in the solutions used were low, their inclusion could cause a small shift of the E_{rev} , away from the E_{H} . Another complicating factor is that the solutions used for these experiments contained Cl⁻; the presence of native HEK-293T Cl⁻ channels (that are activated by acidic pH [398]) could therefore cause a deviation of the E_{rev} of the current. In addition, any permeability of Cl⁻ through the opsins themselves would cause a deviation of the E_{rev} .

Dissecting the contribution of the native currents to the E_{rev} observed in our experiments could potentially be done using single-channel recordings in lipid bilayers where the only current present would be that mediated by the opsins. From these experiments one could elucidate the H⁺ and Cl⁻ permeation through iChloC and ChR2(H134R). However, the low conductance of ChR2 ($\sim 1\text{ pS}$ [306]) could perhaps make these studies rather difficult (as the signal to noise ratio would be low), unless multiple channels were incorporated into the bilayer.

6.3.3 Anion selectivity of iChloC and ChR2(H134R)

As seen from the selectivity experiments in Figure 6.5, in Cl⁻ containing solutions, iChloC mediated currents demonstrated an E_{rev} closer to the E_{Cl} than the ChR2(H134R) expressing cells. This is indicative of an increase in selectivity of iChloC for Cl⁻, compared to ChR2(H134R).

Changing the primary extracellular anion from Cl⁻ to gluconate significantly shifted the E_{rev} of the current observed in iChloC expressing cells towards the more positive calculated E_{Cl} . Generally, gluconate does not permeate native anion channels well compared to Cl⁻ [476]. The results shown here indicate that iChloC mediates a Cl⁻ current but not a gluconate current.

Replacing extracellular Cl⁻ with gluconate shifted the E_{rev} of ChR2(H134R) current by 8 mV towards negative values. This shift does not coincide with the expected shift in E_{Cl} due to the removal of extracellular Cl⁻. It is possible that there is some gluconate permeation through ChR2(H134R) channels.

The E_{rev} of iChloC did not exactly coincide with the E_{Cl} . This discrepancy could be due to the contribution of the E_{rev} of the other permeant ions (e.g. Na⁺, K⁺, Ca²⁺) present, which leads to the E_{rev} of the macroscopic current not overlapping with the E_{Cl} . Solutions in which all permeant ions apart from the anion were substituted or removed could aid in the dissection of the anion current.

6.3.4 Conclusion

iChloC represents a promising opsin for vascular optogenetics. In vascular smooth muscle, iChloC could potentially mimic the native depolarising effect provided by endogenous Cl⁻ channels such as CaCCs coded by the *TMEM16A* gene. Furthermore, the fact that iChloC is H⁺ impermeable means that the dramatic changes in pHi associated with ChR2(H134R) activation (at high light stimulations) would not occur. The slow time course of activation of iChloC would not be a limitation in vascular optogenetics because smooth muscle response to depolarisation is slower than the time course of activation of iChloC. In summary, the data presented in this chapter support the proposal of generating a new mouse

line expressing iChloC in smooth muscle. This line would constitute a new tool for vascular research.

Chapter 7

Summary and conclusions

The overall aim of this thesis was to characterise channelrhodopsins for use in vascular optogenetics. The generation of mice expressing the ChR2(H134R) variant, in conjunction with a series of electrophysiological, optical and molecular biological techniques, has enabled the elucidation of the mechanisms that links ChR2(H134R) activation with arterial contraction. The main findings of this thesis are:

- 1) *Heterologous expression of ChR2 and ChR2(H134R) in HEK-293T cells gave rise to blue light activated currents with similar electrophysiological characteristics, but different level of whole cell current densities.*

ChR2 and ChR2(H134R) currents were long-lasting and non-inactivating. These are electrophysiological features that are compatible with their use in vascular optogenetics. This is because SMC contraction typically develops slowly (over several minutes). Thus, it was argued that light activated currents should also be maintained for several minutes to provoke SMC contraction.

ChR2 and ChR2(H134R) channels share very similar sensitivity to blue light as well as similar kinetic properties. However, ChR2(H134R) elicited overall larger whole-cell currents, possibly due to an increased number of channels expressed on the membrane of the HEK-293T cells. These observations highlight ChR2(H134R) as especially suitable for vascular optogenetics as larger currents would more likely cause V_m depolarisation in SMCs.

Application of optogenetics in vascular research as a tool to define vascular function might require the combined use of pharmacological agents. Thus, in this thesis, analysis of the sensitivity of ChR2(H134R) to a series of ion channel blockers commonly used as research tools was also carried out. To the best of my

knowledge, this is the first report of a pharmacological study of ChR2(H134R). It was found that ChR2(H134R) was not significantly affected by any of the blockers tested.

2) Expression of ChR2(H134R) in vascular SMCs in mice (ChR2(H134R)-SM mice) made the arteries of these mice sensitive to blue light: exposure to blue light led to depolarisation and contraction.

For the first time, a systematic analysis of the blue light sensitivity of SMC from various artery types obtained from ChR2(H134R)-SM mice was performed. Attention was directed to three different artery types: large conduit (aorta), small systemic (mesenteric) and small pulmonary arteries. The amplitude of the currents in all these SMC types could be finely controlled by varying the intensity of the blue light. However, the overall current density was of significantly different in amplitude in the various cell types tested. This was due to different levels of channel expression in the various cell types. In spite of this, all artery types contracted in response to blue light to a level that was comparable to the maximal contraction caused by elevated extracellular KCl. Extending this study to measure the ability of the ChR2(H134R) induced contraction to alter blood flow would be of significant use to establish whether this model could indeed be used to induce ischaemia.

In the aortic SMCs, the study was extended to investigate in detail the relationship between blue light intensity and extent of V_m depolarisation. It was found that ChR2(H134R) opening led to V_m depolarisation from resting values to values corresponding to the peak of the LVGCC window currents. Thus, ChR2(H134R) can serve as a mechanism to bring the V_m to level that can prominently activate

LVGCCs. Extended exposure to blue light led to maintained inward currents and V_m depolarisation.

3) The ionic mechanisms that link ChR2(H134R) opening with muscle contraction have been defined.

Application of the LVGCC blocker nifedipine prevented contraction of isolated aortic rings. However, at high light intensities full contraction was still observed suggesting that Ca^{2+} entry through ChR2(H134R) itself can provide a sufficient increase in $[Ca^{2+}]_c$ to activate the contractile machinery. Using specific pharmacological blockers, CaCCs were also found to be involved in ChR2(H134R)-mediated contraction. CICR, through RyR activation, was not recruited by Ca^{2+} influx via ChR2(H134R). However, the IP₃R blocker 2-APB affected the aortic contraction in response to blue light, implicating these channels in the blue light response. The TRPM channel blocker clotrimazole also strongly reduced the response of isolated arteries to blue light. A major deleterious effect of ChR2(H134R) activation was on the intracellular pH, which was dramatically alkalinised by 0.3 pH units, possibly as direct result of outward H^+ fluxes through ChR2(H134R).

4) A Cl⁻-selective, H⁺-impermeable ChR2 variant (iChloC) may constitute a better opsin for vascular optogenetics.

To find a potential solution to the Ca^{2+} and H^+ permeability of ChR2(H134R) I also examined the photocurrent amplitude, stability, and ability to alter the V_m of iChloC, a reportedly Cl⁻ selective ChR2 variant. iChloC proved to be a highly light sensitive optogenetic tool that mediated large depolarising currents. The increased light sensitivity of iChloC compared to ChR2(H134R) may allow deeper, more uniform penetration and stimulation of tissue but could also limit the tunability of the V_m

control. Selectivity studies showed that iChloC was not proton permeable, thus making it a good candidate for vascular optogenetics.

Future studies

Applying optogenetics to the vasculature has the potential to be of major benefit for the study of vascular physiology and pathophysiology. As outlined in the discussion of Chapter 5, optogenetics has many potential applications in the study of vascular function. These include: 1) the control blood perfusion to different organs or tissues, to induce ischaemia for experimental purposes and 2) investigate the electrical coupling between different cell types within the artery wall. However, ChR2(H134R) opening gives rise to alterations in the intercellular ionic composition (especially the H^+ and Ca^{2+} concentration). These unwanted effects may influence the interpretation of experimental results involving vascular optogenetic because H^+ and Ca^{2+} homeostasis are essential regulators of crucial cellular functions. My data suggest that expression of iChloC in SMC of experimental animals may allow control of vessel tone in the absence of alteration of H^+ and Ca^{2+} dynamics. This model could be generated as part of a future project.

In a clinical setting optogenetics could be used in the treatment of vascular conditions such as hypertension. In this context, a light activated K^+ selective channel could lead to vessel relaxation and help lower the blood pressure. Generating a mouse model with such a channel could allow testing this hypothesis. However, as mentioned in the Introduction, the mutagenesis of ChR2 has, at best, resulted in a marginal improvement in the selectivity for K^+ . Another route to generate these channels is by creating fusion proteins such as BLINK-1, that have

been used to silence neurones in zebrafish [313]. The drawback using BLINK-1 is that it operates on a slow time scale. However, as discussed, controlling vascular tone does not require the fast kinetics that the control of neuronal firing does; thus, BLINK-1 could be a potentially useful tool in the development of future models for vascular optogenetics.

Another exciting development is the ability to control of exogenous channels to control cellular functions in a contact-independent manner without the need for light stimulation (and does negating the need for the implantation of micro LEDs). These approaches include “radio-genetics” and “magneto-genetics”. These methods rely on an engineered temperature sensitive TRPV1 channel which is associated with iron oxide nanoparticles or overexpressed ferritin. Applying a magnetic field or radio waves generated heat in the engineered TRPV1 complex which activated the channel and thus Ca^{2+} influx [477–479]. Through the use of these methods it could be possible to control cellular functions while bypassing the problems associated with uniformly, and deeply, stimulating conventional optogenetic tools.

Concluding remarks

The work presented in this thesis constitutes a comprehensive analysis of the biophysical properties of ChR2(H134R) and its suitability to vascular optogenetics. I also pioneer the idea that a Cl^- -selective and Ca^{2+} and H^+ -impermeable variant may be the most suitable tool for vascular optogenetics. This doctorate project has enabled me to develop a novel tool for vascular research, while also acquiring skills in a variety of modern research techniques. During my doctorate, and in collaboration with my team members, I have also examined the role of endogenous channels, such as CaCCs, in vascular smooth muscle. Although this work has not

been included in the text of the thesis, working on these collaborative projects has allowed me to master new approaches and gain deeper knowledge on vascular ion channels and two peer-reviewed papers have been published as a result of this collaborative research effort. I will be looking forward to participating in and observing future developments in vascular research, especially how my optogenetic approach will aid new discoveries in this fascinating field of the biomedical sciences.

Publications, posters and oral communications

The work in this thesis has been presented at national and international meetings during the course of this DPhil. Furthermore, during my DPhil, I have co-authored two papers published in peer-reviewed primary journals.

Publications

Rorsman NJG, Ta C M, Garnett H, Swietach P, Tammaro P. Defining the ionic mechanisms of optogenetic control of vascular tone by channelrhodopsin-2. *British Journal of Pharmacology* (under review).

Ta CM, Acheson KE*, **Rorsman NJG***, Jongkind RC, Tammaro P. Contrasting effects of phosphatidylinositol 4,5-bisphosphate on cloned TMEM16A and TMEM16B channels. *British Journal of Pharmacology*. 2017 Sep;174(18):2984-2999.

* Denotes equal contribution.

Ta CM, Adomaviciene A, **Rorsman NJG**, Garnett H, Tammaro P. Mechanism of allosteric activation of TMEM16A/ANO1 channels by a commonly used chloride channel blocker. *British Journal of Pharmacology*. 2016 Feb;173(3):511-28.

Posters

Rorsman NJG[#], Ta CM, Swietach P, Tammaro P. Calibrating the optogenetic control of artery tone by channelrhodopsin-2, Channels and Transporters, 15-20th May 2017, Erice, Italy

Rorsman NJG[#], Ta CM, Swietach P, Tammaro P. Developing Channelrhodopsin-2 for use in vascular optogenetics, MSDTC day, 29th of May 2015, Oxford, UK

denotes presenting author.

Oral communications

Rorsman NJG[#]. Establishing optogenetics in vascular pharmacology. Pharmacology 2016 Annual Meeting (London, UK, 15th of December 2016).

Presentation awarded with the Research & Development, Qatar Foundation Oral Communication Prize for best basic oral presentation.

Rorsman NJG[#]. Potentials and limitations of vascular optogenetics. BPS focussed meeting “Pharmacological aspects of microvascular cell-cell signalling and CVS disease” (Oxford, 22nd of September 2016).

Rorsman NJG[#]. Potentials and limitations of vascular optogenetics. Paton Prize Symposium (Oxford, 16th of June 2016).

Rorsman NJG[#]. Developing Channelrhodopsin-2 for use in vascular optogenetics. Wellcome Trust Oxion symposium (Oxford, UK, 25th of September 2015).

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